COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

VOLUME XII

COLD SPRING HARBOR SYMPOSIA
ON QUANTITATIVE BIOLOGY

Founded in 1933
by
REGINALD G. HARRIS
Director of the Biological Laboratory
1924 to 1936

The Symposia were organized and managed by Dr. Harris until his death. Their continued usefulness is a tribute to the soundness of his vision.

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Nucleic Acids and Nucleoproteins

THE BIOLOGICAL LABORATORY
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FOREWORD

When *Nucleic Acids and Nucleoproteins* was chosen as the topic of this year's symposium, I was aware that a similar topic had been discussed only a year earlier at the meeting organized by the Society for Experimental Biology held at Cambridge University. Very few American scientists were able to attend the Cambridge meeting, however, so that an important segment of current research was not reported. This, together with the fact that research in this field is very active and significant new discoveries had been arrived at since last summer, made the selection of this topic for our symposium an appropriate one—particularly since it complements the subjects of our 9th and 11th symposia (*Genes and Chromosomes* and *Heredity and Variation in Microorganisms*) and helps to round out the series of meetings dealing with the mechanism of heredity.

For greater ease of reference, the papers in this volume are arranged alphabetically according to authors. On the program they were arranged in what appeared at the time to be a logical sequence according to subject matter. The first group of papers to be presented dealt with the chemical aspects of the problem (Greenstein, Carter, and Chalkley; Schmidt, Cubiles, and Thannhauser; Gulland; Michaelis). These were followed by Spiegelman and Kamen's paper on the role of nucleoproteins in enzyme formation, and by Taylor, Greenstein, and Hollaender's paper on the effect of X-rays on nucleic acid. Certain aspects of the behavior of nucleic acids in living cells and tissues were elaborated in three papers given by Davidson, Thorell, and Brachet. This year's program placed considerable emphasis on the conditions existing in microorganisms and on work using microorganisms as experimental material; six papers dealt with research involving viruses and bacteria (Knight, Hyden, Cohen, Chargaff, Boivin, and Witkin). Another important part of the program was devoted to papers dealing with the nucleus and with chromosomes. This group included the contributions of Stedman and Stedman; Mirsky; Pollister and Ris; Schultz; and Mazia, Hayashi, and Yudowitch. The sessions were concluded with a paper by Schneider on nucleic acids in normal and neoplastic tissues.

Owing to unfortunate circumstances which the efforts of the Laboratory could not overcome, Belozersky and Serra were not able to attend the symposium. Their papers are included in this volume, however.

The publication of discussions in this volume is according to the procedure followed for the last two symposia. Participants were requested to submit manuscripts covering their questions, comments, or statements if they felt these represented a significant contribution. Only the material so received has been included in the volume.

Material presented by Errera and by Ris during the discussions contained new experimental details, and their contributions are published here as separate papers.

In the organization of the program for this symposium I was helped by Drs. J. P. Greenstein, A. Hollaender, M. McDonald, and A. Mirsky; I wish to express to them my sincere appreciation. The editing was done by Dr. Katherine Brehme Warren.

The symposium was held from June 11 to June 20, 1947. The meetings were attended by about 150 persons. It is my pleasure to acknowledge here a grant from the Carnegie Corporation, which enabled us to bring scientists from abroad to our meetings.

It was with deep regret that we learned of the death in October of our colleague, John Masson Gulland. He came to us from England to give one of the most significant papers of the Symposium, and his warm personality, as well as the excellence of his work, made many friends for him in America. Since his death occurred so soon after our meeting, which was one of the last that he attended, I feel that it is appropriate that we reproduce here a photograph of Dr. Gulland and a brief biographical statement prepared by one of his colleagues, and that we dedicate this volume to one whose life work and interest were concerned with problems that were discussed at the Symposium.

M. DEMEREC

JOHN MASSON GULLAND

F.R.S., M.A. (Oxon.), PH.D. (St. Andrews), D.Sc. (Edin.)

JOHN MASSON GULLAND was born in 1898 in Edinburgh, Scotland, son of Professor G. L. Gulland of Edinburgh University. He went to school and University in Edinburgh, but his student days were interrupted by the first World War, during which he served as a 2nd Lieutenant in the Royal Engineers from 1917 to 1919. He took his B.Sc. degree in 1921 and later did research work in St. Andrews and Manchester. In 1924 he went to Oxford, where he remained until 1931 when he was appointed Reader in Biochemistry in the University of London at the Lister Institute. Five years later he was appointed Sir Jesse Boote Professor of Chemistry in University College, Nottingham. During the second World War he acted as Assistant Director, Chemical Research and Development, Ministry of Supply, from 1943 to 1944; and he had just taken over a new post as Director of Research to the Institute of Brewing when he was killed in a railway accident on 26th October, 1947. He is survived by a widow and two daughters.

Gulland's interests ranged over a wide field of Chemistry and Biochemistry, and his early work dealt with alkaloids. During his stay at the Lister Institute he was engaged on the difficult problem of the pituitary hormones. But it is from his work on the nucleic acids that he will be chiefly remembered. For nearly 20 years he had been engaged in investigating the structure of the nucleic acids and of their constituent nucleotides, and much of our present-day knowledge of the chemical constitution of these substances has come from Gulland's laboratory.

Gulland was one of the most courteous and charming of men. The invitation to attend the Cold Spring Harbor Symposium on nucleic acids gave him great pleasure, and during his visit to the United States he made many friends who have learned with sorrow of the tragedy which has withdrawn from the field of nucleic acid chemistry one of its foremost investigators.

J. N. DAVIDSON

J. Masson Gulland.

Photograph by J. Russell and Sons, London, by courtesy of the Royal Society

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ON THE NUCLEOPROTEINS AND POLYNUCLEOTIDES OF CERTAIN BACTERIA

A. N. BELOZERSKY

Bacteria are characterized by an extraordinarily high content of proteins and nucleic acids. It is hardly possible to find in the plant kingdom any other organisms which contain in their cells such a large amount of proteins and nucleic acids. In this respect bacteria occupy a quite special place not only among plants but also among animals. The predominance of these substances in the bacterial cell becomes particularly clear if a comparison of bacteria be made with others of the lower organisms, as well as a comparison with the reproductive cells and embryonic tissues of the higher plants, that is, with tissues and cells, in which proteins and the nucleic acids are accumulated in particularly great amounts.

In Table 1 are to be found comparative data on the content of proteins and nucleic acids, calculated from the determination of total nitrogen and nuclein phosphorus and expressed in percent of dry weight for bacteria, other microorganisms, reproductive cells and embryonic tissues.

The data represented in Table 1 may serve as a good illustration of the exceptional richness of the bacterial cell in proteins and nucleic acids. It is important to note that the quantity of nucleic acids varies depending upon the age of the bacterial culture. One may suppose that the accumulation of great quantities of these important substances is one of the factors responsible for the exceptional vitality of the bacterial cell, which finds expression in very intensive metabolism, rapidity of reproduction and remarkable adaptability to the environment.

The evidence indicates that the greater part of proteins in the bacterial cell is bound up with the polynucleotides to form nucleoproteins. Nucleoproteins from bacteria can be but poorly extracted and in most cases it appears that they cannot be extracted at all, at least by such solvents as water and salt-solutions. The easiest method of their extraction is by application of dilute alkaline solutions. For this purpose it is possible to use sodium carbonate or even preferably a 0.2% solution of sodium hydroxide. However, even when using dilute alkali solutions it is sometimes impossible to extract nucleoproteins from a great many bacteria. This may be explained by the peculiar composition and structure of the cell wall of some bacteria. The application of strong alkaline solutions as well as preliminary autolysis of the bacterial mass in the course of nucleoprotein extraction, as done by certain investigators, is fraught with risk, because this method often leads to a partial destruction of the nucleoproteins and contributes to inaccuracy of the results.

The high polynucleotide content of bacteria has been found not only from determinations of the nuclein phosphorus, but also from the actual yields of free polynucleotides and products of a nucleoprotein type. Thus, for instance, from the cells of young cultures of *Spirillum volutans* we succeeded in isolating as much as 10% to 12% of the dry weight of bacteria polynucleotide of the yeast type (Belozersky, 1941). The nucleoproteins isolated directly from bacteria do not represent individual constituents formed in the cell, and as a rule, may be fractionated into a number of products differing in their chemical nature and undoubtedly also in their physiological function. The chemical fractionation method applied by us for this purpose both for nucleoproteins to be extracted from the higher plant tissues (Belozersky, 1936; Belozersky and Dubrowskaja, 1936; Belozersky and Chernomordikova, 1940; Belozersky, 1941; Belozersky and Uspenskaja, 1942) and for bacterial nucleoproteins (Belozersky, 1941; Belozersky, Korneeva and Pomostchnikova, 1947; Peshkov, Belozersky, Korneeva and Karpouchina, 1947) cannot be regarded as beyond criticism, since in this case we are forced to work with strong reagents. However, in spite of this difficulty, the method of chemical fractionation has played a large part in our work. Indeed, the application of this method has allowed us to prove experimentally the non-individuality of the nucleoproteins extracted from the plant cells and tissues, and to ascertain their undoubtedly secondary formation at the moment of isolation. Further, we succeeded in establishing the fact that the protein and the nucleic acid in the nucleoproteins may be bound in different ways. And finally, the most interesting result of the chemical fractionation method consisted in our obtaining several different fractionation products.

In fractionation of nucleoproteins separated with the help of alkali extraction, we succeeded in obtaining from *Spirillum volutans* (1941), *B. paradysenteriae* Flexner and *E. typhosa* (1947), as the final fractionation products, a protein containing only traces of phosphorus, free nucleic acid and a nucleoprotein.

It is interesting to note that analogous products could be obtained by the fractionation of "nucleoproteins" isolated directly from the tissues of higher plants.

Protein predominated quantitatively over the other fractionation products. There was good reason to suppose that the given protein was a cytoplasmic protein.

The free nucleic acid formed during the fractionation in this case as well as in the case of higher plants, was as a rule a nucleic acid of the yeast type. We usually established this by determining its pentose content and investigating its hydrolysis products. In rarer cases, side by side with a pentosepolynucleotide of the yeast type there could be found in this fraction small amounts of free thymonucleic acid. At all events, under the prevailing conditions of fractionation the pentosepolynucleotides were always completely separated from the proteins while the thymonucleic acid was separated only partially, its yield in the free state in the same material depending upon age and the physiological condition of the bacterial culture.

Pentosepolynucleotides of the yeast type as well as the form of thymonucleic acid isolated during the fractionation seemed to be found in the cells in a labile, salt-like combination with the protein. Such a view followed from the possibility of separating them from the protein by fractionation and also from our model experiments for obtaining synthetic nucleoproteins from cytoplasmic protein and this nucleic acid followed by fractionation (Belozersky, 1936a; 1940b). Depending upon conditions it was possible to assume the production of salts in varying proportions, formed from the protein and nucleic acid. This supposition follows from the fact that nucleic acid may be bound with protein only to a certain limit. Below this limit it is possible to obtain nucleoproteins with different relative proportions of protein and nucleic acid. Our model experiments with substituted non-basic protein have shown that if we consider which protein groups react with nucleic acid, it may be said that amino groups are liable to react because proteins with blocked amino groups completely lose their ability to bind nucleic acid (Belozersky and Bajilina, 1944a). These facts gave us the right to suppose that under biological conditions, dependent upon the environment, qualitatively and quantitatively different amounts of nucleic acid may combine with the amino groups of the cytoplasmic proteins, which naturally influences their functional manifestations.

The nucleoprotein obtained as a consequence of the fractionation of the original "nucleoprotein" was of course a real nucleoprotein preformed in the cell. This nucleoprotein may be more exactly called a nuclear nucleoprotein, because as a nucleic acid it contained a thymonucleic acid, with complete absence of pentosepolynucleotides of the yeast type. The thymonucleic acid was identified by the author in the nuclear nucleoproteins from *B. paradysenteriae* Flexner and *E. typhosa* and in the nuclear nucleoproteins from a number of higher plants (Belozersky, 1936; Belozersky and Dubrowskaja, 1936; Belozersky and Tshigirjev, 1936; Belozersky and Chernomordikova, 1940; Belozersky and Uspenskaja, 1942), this being done not only on the basis of the Feulgen reaction, but also on the basis of isolation and identification of all the purine and pyrimidine bases characteristic of thymonucleic acid. In a number of experiments we succeeded in isolating levulinic acid, which is usually formed from desoxyribose during acid hydrolysis. In the case of *Spirillum volutans* in the nuclear nucleoprotein we found not a thymonucleic acid, but a new nucleic acid, containing all the purine and pyrimidines including also thymine, but which in addition to desoxyribose contained other pentose. Quantitative determination of pentose showed that in this case the molecule apparently contained two pentose and two desoxyribose residues. It is possible that in this hybrid nucleic acid the pentose is bound up with purines while desoxyribose is associated with pyrimidines. In consequence a weak Feulgen reaction was observed in the cells of *Spirillum volutans*, as well as in the nucleoprotein preparations obtained from them.

The fractionation process may be represented as follows:

the original "nucleoprotein"
obtained as a result of extraction with dilute alkali and precipitation with acid

↓ *Cytoplasmic protein*

↓ intermediate product

↓ *Nucleoprotein* (protein + thymonucleic acid)

↓ *Free nucleic acid* (of yeast type, in rare cases with added thymonucleic acid)

TABLE 1

Organism	Nucleic acids	Proteins	Authors
Micrococcus candicans	14.2	53.3	Belozersky, (1940)
Staphylococcus albus	17.4	54.9	Belozersky, (1940)
Sarcina lutea	11.9	63.7	Belozersky, (1940)
Sarcina flava R-form	10.4	66.6	Belozersky, (1946)
Sarcina flava S-form	10.3	65.3	Belozersky, (1946)
B. pyocyaneum	21.6	48.8	Belozersky, (1940)
B. fluorescens liquefac	14.0	54.8	Belozersky, (1940)
B. mycoides	11.4	52.4	Belozersky, (1940)
B. megatherium	10.2	49.2	Belozersky, (1940)
B. aceto-aethylicum	10.3	49.8	Belozersky, (1940)
Proteus vulgaris	13.0	47.0	Belozersky, (1940)
Mycobacterium luteus	14.4	42.9	Belozersky, (1940)
Myxobacterium sorangium sp.	12.6	37.4	Belozersky, (1940)
Spirillum volutans			
2-days old culture	28.8	36.4	Belozersky (1941)
4-days old culture	21.3	48.6	Belozersky, (1941)
6-days old culture	10.6	63.6	Belozersky, (1941)
E. coli			Belozersky, Korneeva and Karpouchina (1947)
5-hours culture	22.43	57.0	
20-hours culture	14.13	72.4	
48-hours culture	9.66	70.4	
B. paradysenteriae Flexner			Belozersky, Korneeva and Karpouchina (1947)
5-hours culture	28.18	61.0	
20-hours culture	19.97	74.0	
48-hours culture	14.77	70.9	
E. typhosa	12.1	64.1	Belozersky, Korneeva and Pomostchnikova, (1947)
Plasmodium reticularia lycoperdon	3.68	29.07	Kiesel (1929)
Yeast	5–10	37.5–50.0	Terroine and Szucs (1930)
Sterigmatocystis nigra		25.8–39.5	Terroine and Szucs (1930)
Aspergillus oryzae	3–6	24.62	Terroine and Szucs (1930)
Penicillium glaucum		32.54	Terroine and Szucs (1930)
Fuligo varians (spores)	7.9	40.6	Kiesel (1929)
Germs of cedar seeds	6.80	26.10	Belozersky and Uspenskaja (1942)

Experience has shown that it is not always possible to fractionate in the above manner the "nucleoproteins" obtained directly from a bacterial mass. For instance, we could not fractionate the "nucleoprotein" from *Proteus vulgaris*. However, on the basis of isolation and identification of purine and pyrimidine bases from the hydrolysate of a given nucleoprotein it was possible to conclude that the cells of *Proteus vulgaris* contained a pentosepolynucleotide of the yeast type and also thymonucleic acid (Belozersky, 1939). In *Myxobacterium sorangium* sp., we found both types of polynucleotides (Belozersky, 1939). From *Sarcina flava* (Belozersky, 1946) and *Sarcina lutea* (Belozersky and Kireenkova, 1943) the yeast polynucleotide was completely isolated by extraction with dilute alkali, while at the same time the thymonucleic acid remained unextracted in the residue. We accomplished the identification of these two types of polynucleotides by way of qualitative determination of the carbohydrate components and also by isolating and identifying the respective purine and pyrimidine bases.

In the nuclear nucleoproteins of bacteria and in those of higher plants, the thymonucleic acid is as a rule closely bound with protein. This follows from the inability of nuclear nucleoproteins to be fractionated into their components. In some cases, however, by means of fractionation, as shown above, we succeeded in separating from a nuclear nucleoprotein a greater or lesser amount of thymonucleic acid. The variable nature of the correlation between the components in the nuclear nucleoproteins is undoubtedly connected with the physiological state of the cell. In the cells of young bacterial cultures one may observe an increase in the absolute amount of nuclear nucleoprotein, an increase in the content of thymonucleic acid and a predominantly close bond between the components of the nuclear nucleoprotein. With the aging of the culture a decrease in the absolute quantity of the nuclear nucleoprotein and of the content of nuclear nucleic acid is observed, while at the same time the last partially passes into a state in which there is a loose bond with the protein. We had occasion to observe particularly well this kind of change in *Spirillum volutans* (Belozersky, 1941).

The above mentioned facts brought us to the

conclusion that thymonucleic acid or acids of a similar kind must be regarded as a potent factor in determining the integrity of the pattern and composition of the specific protein of a given species (Belozersky, 1941, 1944b). In fact, in the literature there are to be found a great many facts, testifying to the inconstancy of the protein molecule, in the sense that its composition and consequently also its pattern depend not only upon the state of development of the organism, but also upon the condition of the latter. We believe it to be plausible that the thymonucleic acid or a similar acid entering into a close combination with the protein excludes it from the sphere of active reactions and thereby seems to fix and to fasten the pattern and composition of the particular protein. Maximum blocking takes place during cell division. Externally this is expressed both in an increase in the amount of nucleic acid and in its transition to a considerable degree into a state of close bonding with the protein. The successive decrease of the amount of thymonucleic acid, accompanied by a partial passage into a state of loose combination with the protein, corresponds to the partial unblocking of the protein.

Bacterial cells are characterized by their high content of nuclear substance. As is shown by our analytical data, as well as by the direct method of isolation, the amount of the nuclear substance in different bacteria varies from 20% to 40% of the dry weight of the bacteria. These data allowed us at one time to favor the existence of a diffuse nuclear apparatus in bacteria (Belozersky, 1944a). In fact, it is difficult to imagine how a vast number of first-rate cytologists, whose work is technically excellent, could have made such a mistake as to leave undiscovered the presence in the bacterial cell of morphologically differentiated formations, which are undoubtedly sufficiently large to be observed.

In recent years in the USSR (Peshkov) and in England (Robinow) showed the presence of a well-formed cell nucleus in a number of bacteria. Nevertheless, even to the present moment there are some microbiologists and cytologists who support the idea of a diffuse nuclear apparatus in the bacteria (Imšenecki, USSR; Knaysi, USA). It is to be hoped that in the near future certain difficulties in the cytological investigation of bacteria will be surmounted and the question of the bacterial nucleus will find a satisfactory solution.

Concerning the chemical nature of the protein component of the nuclear nucleoproteins and bacterial proteins in general, our data on the content of basic amino acids shown in Table 2 and expressed as dry weight of the preparation testify to the absence of proteins of the basic character, of the histone type and of course also of protamines in the bacteria (Belozersky, 1939; Belozersky, 1941; Belozersky and Kireenkova, 1943; Belozersky, Korneeva and Pomostchnikova, 1947; Peshkov, Belozersky, *et al.*, 1947). This conclusion refers in the same degree to the nuclear proteins of the higher plants. It is impossible to draw a conclusion about the proteins of the histone type from the high nitrogen content of the basic fraction of the hydrolysate as has been done by some authors, because the bacteria and the bacterial nucleoproteins contain a great quantity of nucleic acids, whose decomposition products usually greatly increase the content of nitrogen in the basic fraction of the hydrolysate.

TABLE 2

Object of investigation	Arginine	Histidine	Lysine
Nucleoprotein from *Proteus vulgaris*	3.83	2.04	6.38
Nucleoprotein from *Spirillum volutans*	4.71	1.83	6.20
Cytoplasmic protein from *Spirillum volutans*	4.63	1.75	5.91
Nucleoprotein from *E. typhosa*	4.60	0.30	4.50
Nucleoprotein from *B. paradysenteriae* Flexner	1.96	0.21	2.77
Cytoplasmic protein from *B. paradysenteriae* Flexner	5.22	0.43	4.24
Bodies *B. Coli*	6.65	0.37	3.28
Bodies Myxobacterium Sorangium sp.	3.68	1.43	4.22
Bodies *Sarcina lutea*	2.32	0.40	2.24
Bodies *Sarcina flava* S-form	2.95	0.05	4.72
Bodies *Sarcina flava* R-form	3.13	0.07	4.62
Nucleoprotein from soy bean seedlings	4.49	2.04	6.77
Nucleoprotein from wheat germs	3.19	1.18	3.80
Nucleoprotein from cedar seed germs	8.20	2.60	6.20

Beginning with the year 1936 we have always emphasized this circumstance. In 1942, in our work (Belozersky and Uspenskaja) on the nucleoproteins from the cedar nut embryo, we wrote: "These data allow us once more to express our opinion as contrary to the prevailing point of view, widespread among biochemists and biologists investigating the cell nucleus, to the effect that thymonucleic acid in the nucleus must be bound up with proteins of a basic character belonging to the type of histones or protamines."

If we have no evidence for denying the presence of histones in plant cells and in bacterial cells, we at any rate have reason to maintain that it is proteins of high molecular weight, characterized by their qualitative multiformity of amino acids and complexity of structure ensuing from the qualitative diversity of their amino acid composition, that are the most typical and specific constituents of the plant cell nucleus.

In our opinion histones and protamines should be

considered as auxiliary proteins, even in spite of their quantitative preponderance in some animal nuclei, because it is difficult to conceive how it would be possible for a reproduction of proteins, complex in their composition and structure, to take place on the basis of simple proteins of the histone type and particularly of the protamine type.

It is quite natural that we met with great satisfaction the work of Stedman and Stedman, dealing with chromosomin in the nucleus of animal cells.

As we have shown in Table 1, the quantity of polynucleotides varies greatly, depending on the age of the culture. This was established by us for *Spirillum volutans, B. paradysenteriae* Flexner and *B. coli.* We subjected the two latter cultures to a detailed study from the chemical point of view with the collaboration of our co-workers, Korneeva and Karpouchina. At the same time, these two cultures at the age of 5-, 20-, and 48 hours respectively were investigated cytologically by Peshkov. By our comparison of cytological data with chemical data, we established a number of interesting facts (Peshkov, Belozersky and others, 1947). First of all it appeared that strongly basophilic cells of cultures aged 5 hours contain 2 to 3 times as much pentosenucleic acids as the cells in cultures 20 to 48 hours old, in which basophilia was very weak or was quite absent. Detailed chemical investigation showed that the basophilia of young bacterial cells is always associated with pentosepolynucleotide of the yeast type. In this case the pentosemononucleotides could not be wholly responsible. Although their content in the young cells was also much higher, yet their total amount was low in comparison with the amount of pentosepolynucleotides. It is important to note that apparently in the cytoplasm of the bacterial cell one must distinguish between two types of pentosepolynucleotides. The first is in a close contact with the cytoplasmic protein, while at the same time the second apparently is not bound with protein. Basophilia is apparently conditioned by this second form of pentosepolynucleotides. We made this conclusion on the basis of the fact that that nucleoproteins obtained by way of precipitation from extracts of the 5-, 20-, and 48 hours old bacterial cultures were characterized by approximately the same content of pentosepolynucleotides. Quite another picture was observed with filtrates after the precipitation of nucleoproteins. A pentosepolynucleotide could be found only in a filtrate of a 5 hour old culture. At the same time its amount exactly corresponded to the excess which is characteristic for a young basophilic culture.

Analogous results were earlier obtained by us with the culture of *Spirillum volutans* (Belozersky, 1941). From a young culture two days old, rich in volutin, we succeeded in isolating as much as 10% to 12% of the dry weight of bacteria as pentosepolynucleotide, while from a 6 day old culture entirely deprived of volutin it was quite impossible to isolate the respective fraction of pentosepolynucleotide. Meanwhile the nucleoproteins isolated from 2 and 6 day old cultures contained approximately the same amount of pentosepolynucleotide. On account of the fact that an enormous quantity of pentosepolynucleotide could be obtained only from a culture rich in volutin, we surmised that the given pentosepolynucleotide appeared to be a voluntin substance. The investigation showed that this pentosepolynucleotide was characterized by a higher phosphorus content than the usual pentosepolynucleotides of the yeast type, while with respect to its purine and pyrimidine content, it corresponded to a yeast polynucleotide. Besides, it was established that it contained sulphuric acid in an esterlike bond. As a result of all data obtained we designated this pentosepolynucleotide as a volutin nucleic acid (Belozersky, 1945).

In the *Spirillum volutans* only the voluntin nucleic acid was subjected to sharp fluctuations. The pentosepolynucleotide of the yeast type, bound with the protein, remained without any change in the 2, 4, and 6 day old cultures. Its amount was always about 3% to 3.5% of the dry weight of bacteria, while the content of volutin nucleic acid dropped from 12% in the 2 day cultures to zero in the 6 day cultures.

Thus, on the basis of the above mentioned data we suppose that the fraction of pentosepolynucleotide, bound up with the protein and subjected to small changes only, appears to be a constitutional component of the protoplasm in the bacterial cell. The variable portion of the pentosepolynucleotide, which is evidently not bound up with the protein, seems to have a quite different physiological significance.

In our study of R and S forms of *Sarcina flava* of the same age, we did not succeed in finding any differences in the qualitative and quantitive composition of the polynucleotide (Belozersky, 1946). In both forms we found that pentosepolynucleotide of the yeast type and thymonucleic acid were present in approximately the same amounts.

Summarizing our experimental data we may note that the cells of different bacteria are very similar in their composition. They are all characterized by a high content of nucleoproteins and polynucleotides.

Bacterial polynucleotides belong to two types: to the type of yeast polynucleotide and the type of thymonucleic acid. On the other hand, our experimental material as well as that described in the literature testifies to the need for a more exact identification of polynucleotides in each separate case, since different variations might be present. In this respect we must fully agree with Gulland, who in his survey of nucleoproteins and nucleic acids (Gulland, Barker and Jordan, 1945) emphasizes the possibility of a structural and compositional isomerism in the polynucleotide group. Some of our observations show the possibility of a different

degree of polymerization of polynucleotides in the bacterial cell.

The study of the isomerism of polynucleotides and of the degree of their polymerization in the cell presents particular interest from the point of view of phylogenetic correlation in the nucleic acid group.

In conclusion we should like to express our profound gratitude to Dr. M. Demerec, who gave us the opportunity of making in the present report a short summary of the investigations of many years in the field of bacterial nucleoproteins.

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DIRECTED MUTATION IN COLON BACILLI, BY AN INDUCING PRINCIPLE OF DESOXYRIBONUCLEIC NATURE: ITS MEANING FOR THE GENERAL BIOCHEMISTRY OF HEREDITY

ANDRÉ BOIVIN

Mutations occur spontaneously in bacteria as they do in higher organisms, animal and plant. It is possible, at least in certain cases, to increase the frequency of these mutations by various physical agents (X-rays, etc.) or by chemical ones. In plants and animals it has not yet been possible to "direct" the process of mutation, that is, to produce from a given genotype another genotype, determined in advance. This, however, can be achieved with bacteria. In a few favorable cases it has been possible to transform an antigenic type of pneumococcus into another type of pneumococcus fixed in advance, or an antigenic type of colon bacillus into another type of colon bacillus, also fixed in advance, by utilizing an inducing principle of a desoxyribonucleic nature, chemically isolated from the bacterium whose genotype is desired.

In the present report we propose:

(1) To review briefly the fundamental observations made by Avery and collaborators on pneumococcus, and to report in detail our own observations on colon bacilli.

(2) To study the participation of desoxyribonucleic acid (and also of ribonucleic acid) in the chemical constitution and the cytological structure of bacteria in general and of colon bacilli in particular.

(3) To discuss the role of desoxyribonucleic acid (and also of ribonucleic acid) in the bacterial cell, and the mechanism by which desoxyribonucleic acid can, after leaving the bacterial cell, induce and direct mutations in other bacilli; more generally, to discuss the role of nucleic acids in all cells.

I. Example of Mutation in Colon Bacillus Directed by an Inducing Principle of Desoxyribonucleic Nature

Almost twenty years ago, Griffith discovered the possibility of transforming one type of pneumococcus into another type, by bringing into contact, *in vivo* or *in vitro*, the living form R (rough) of the type to be transformed, with the *heat-killed* form S (smooth) of the type to be obtained. The same result was obtained by substituting a bacterial extract for the killed bacteria (Dawson and Sia; Alloway). It remained for Avery, to whom immunology already owed a great deal, and to his collaborators McCarty and MacLeod, to discover the chemical nature of the inducing principle in question—namely, the desoxyribonucleic acid of the bacterium which imposes its own type (Avery, MacLeod, and McCarty, 1944; McCarty, 1945; McCarty and Avery, 1946; McCarty, 1946b). This was isolated from pneumococcus in a practically pure state and with a high degree of polymerization (molecular weight of the order of 500,000; solution of its sodium salt very viscous). Its power of inducing mutation is soon inactivated (as soon as depolymerization begins) under the effect of desoxyribonuclease. Strange to say, it will act on a pneumococcus and induce a mutation only in the presence of blood serum or of ascitic fluids, the exact role of which is still very obscure (apparently not from intervention of the antibody R).

The studies which my collaborators and I have made on the colon bacilli have enabled us to discover facts which are entirely comparable.

Before the War (in collaboration with Mesrobeanu and A. and G. Magheru) and especially during the War, in 1942, 1943, and 1944 (in collaboration with Corre and Lehoult) we found evidence of the extraordinary multiplicity of antigenic types among the colon bacilli, each type possessing its own polysaccharide, characterized by a special chemical constitution and by a particular serological specificity. Each type remains stable through successive cultures; like the pneumococcus types, it can undergo antigenic degradation leading from form S (smooth) to form R (rough) by losing its polysaccharide, and, like the pneumococcus types again, it has the value of a true elementary species, within the immense species of *Escherichia coli*. We wished to discover whether, like the pneumococci, the colon bacilli might not give way, by controlled mutation, to the process of type-transformation. For this purpose we have made a large number of experiments with simultaneous cultures of the two types in the same broth or in two portions of the same broth, separated either by a porous membrane (bougie filter) or by a collodion membrane; we have also tried to cultivate one type in a filtrate of culture from another type. In the course of our study we have frequently come across more or less marked antibiotic reactions, which we have not studied further. In one case we observed the transformation of a type, which we studied in detail.

We will therefore report here on the observations made on this subject by my collaborators Delaunay, Vendrely and Lehoult, and by myself (1945 and 1946).

We have worked with two colon bacilli (#17 and #24) isolated, among many other bacilli, from fecal matter of normal subjects. For the sake of convenience we shall designate these two bacilli as C_1 and C_2 and their S and R forms as S_1 and R_1, S_2 and R_2, respectively, as we have done in our previous publications. In their S form these bacilli differ from each other very clearly in the chemical constitution and in the serological specificity of their polysaccharides (no cross precipitation reactions). Chemically, the polysaccharide of S_1 contains approximately one third of its weight in uronic acids and two thirds in neutral sugars, while the polysaccharide of S_2 is built up exclusively of neutral sugars; in both cases the neutral sugars appear to be hexoses, without admixture of pentoses or hexosamines. Spontaneously, in the course of the cultures, S_1 frequently produced R_1, and S_2 produces R_2; but in no case have we observed a spontaneous reversal of R to S, with or without change of type.

Cultured in a filtrate (on Bougie filter) of a broth culture of S_1, R_2 often gives rise to S_1; thus one obtains a mixture of the forms R_2 and S_1, easily distinguishable because of the difference in appearance of their colonies on agar (the classical difference between rough and smooth colonies). S_1, arisen from R_2 (and consequently from S_2) presents all the serological and chemical characters of natural S_1, and, like it, remains stable throughout all successive transfers and is able to produce only a degradation in R_1. The same result may be obtained by cultivating R_2 in broth with the addition of an autolysate of S_1 (bacilli killed by toluene, then exposed for two hours to ordinary heat, in 9/1000 NaCl and centrifuged to eliminate dead bacteria).[1] The active principle is found again in the nucleoprotein fraction which can be precipitated from the autolysate at pH 3.5. By a series of precipitations at this pH, followed by repeated solution in a bicarbonate medium, one finally eliminates the antigenic gluco-lipo-polypeptide complex of S_1 which the crude autolysate contained in abundance (following purification serologically). The active principle is found again in the crude nucleic acid which can be isolated from nucleoproteins by pepsin digestion (a few hours at ordinary temperature and at pH 2), or better by Sevag procedure (chloroform), followed by repeated fractional precipitations with acid (HCl) alcohol at low temperature and as rapidly as possible. Before digestion or before action of chloroform, the preparations precipitate abundantly with serum from a rabbit immunized with nucleoprotein and they contain 4 to 6 times more proteins than nucleic acid. After deproteinisation and fractionation, all serological precipitation has disappeared and one has a substance containing nucleic acid in the amount of from 70% to 90% of its weight. This is a mixture of desoxyribonucleic acid and ribonucleic acid, containing from 40% to 75% of the former, according to the manner in which the fractionations were carried out. The activity of the product (in solution of sodium salt) is very marked at a concentration of 1/1000 and subsides only at a concentration of about 1/100,000 to 1/1,000,000.[2] It resists the action of ribonuclease but disappears very rapidly under the effect of desoxyribonuclease (enzyme isolated from pancreas according to Fischer and collaborators and according to McCarty). Therefore it can be stated that the active principle in question is the desoxyribonucleic acid of S_1, highly polymerized and yielding strongly viscous solutions (it is known that desoxyribonuclease of the pancreas depolymerizes the acid only, without separating the nucleotides). Mutation of the colon bacillus takes place successfully without addition of serum or ascitic fluids, contrary to what happens with pneumococcus. We have established that if one substitute, for nucleic acid of S_1, crude nucleic acids derived from three other colon bacilli, and from staphylococcus, yeast, spleen, and thymus, no results are obtained. It is clear therefore, that not *any* kind of nucleic acid, but only desoxyribonucleic acid derived from S_1, can induce the change from S_2 to S_1. Even crude nucleic acid isolated from R_1 is inactive. This suggests the existence in nature of numerous desoxyribonucleic acids, differentiated by their particular biological

[1] We cannot dissolve bacilli in sodium desoxycholate, as was done by Avery with pneumococcus; in fact, colon bacilli resist this reagent and yield only slight traces of nucleoproteins. The same is true for the use of M NaCl, which gave such good results in the experiments of Mirsky and Pollister with animal tissues; while the pneumococci, brought in contact with NaCl, liberate a small quantity of desoxyribonucleic acid, colon bacilli liberate practically nothing. (Mirsky and Pollister have confirmed this fact recently.)

[2] Despite apparently identical experimental conditions, the transformation of R_2 into S_1 through the action of the desoxyribonucleic acid of S_1 is not regularly produced. In a dozen tubes, containing the same volume of medium and the same dosage of desoxyribonucleic acid, inoculated with the same number of bacteria, one frequently finds tubes giving rise to transformation side by side with others where no transformation occurs. The number of bacteria at the beginning and end of the culture and the concentration of the desoxyribonucleic principle do not allow an explanation on statistical grounds of the proportion of positive results obtained in the different experiments. All takes place as though a factor, still unknown, were able to facilitate or to prevent transformation. An analogous situation is found in toxinogenesis, where one often sees flasks of the same medium, inoculated with the same toxigenic bacteria and grown under the same conditions, which give very unequal yields of toxins. The intervention of this unknown factor prevents the precise determination of the "frequency" of transformation of R_2 into S_1 under the effect of the desoxyribonucleic acid of S_1.

qualities, and consequently also by some details in their chemical constitution: at least one acid for each type of pneumococcus (about 100 types are known), one acid for each type of colon bacillus (there probably exist hundreds or thousands of types of colon bacillus), etc. In reality it is necessary to postulate the existence of a desoxyribonucleic component for every S form, which does not reappear in the corresponding desoxyribonucleic acid of form R. We shall come back to these significant facts in more detail.

We had already come across the phenomenon of type transformation in colon bacilli, and had recognized the activity of nucleoprotein preparations obtained by autolysis, when the fundamental work of Avery and his collaborators (1944), revealing the role of desoxyribonucleic acid, was brought to our attention. Inspired by this work, we too have obtained evidence of the intervention of desoxyribonucleic acid in directed mutations in bacteria. We take pleasure in acknowledging the priority of the American authors in this field.

As might be supposed, we tried to reproduce the reverse mutation, that is, to pass from R_1 to S_2—using an extract of S_2. We repeatedly failed, however. It is quite probable that the mutation of a bacillus under the influence of desoxyribonucleic acid from another bacillus (closely related to the first) is always *potentially* possible, but that the frequency with which it is effectively produced can vary within considerable limits from one case to another. However, the fact that mutant S gives rise to a diffuse culture, invading the medium, while the original form R grows at the bottom of the tube in small granules, is of great assistance in isolating S in the presence of R.

It is evident that the various colon bacilli colonies show a very unequal capacity to mutate. While C_2 passes from S to R, one obtains an entire "spectrum" of R_2 variants, differing more or less from each other in the appearance of their colonies; and only one of them, a variant with very small colonies, has, up to now, shown itself capable of changing from C_2 to C_1. Avery has encountered entirely comparable phenomena with pneumococcus.

We have summarized all the facts reported above in the following diagram:

The change from C_2 to C_1 evidently requires a certain rearrangement of the enzymatic equipment of the bacillus, since C_1 produces a polysaccharide which is different from that of C_2. But it seems that more extended modifications of the enzymatic equipment must take place: S_1 original, and S_1 derived from R_2, do not ferment sucrose, while S_2 does act upon sucrose. Is the mutation from C_2 to C_1 accompanied by a loss of capacity to ferment sucrose? We thought so, and so stated it at the outset of our researches. Facts which we have observed since then, however, have somewhat obscured the question; the different variants of R_2 show themselves unequally capable of attacking sucrose and, besides a variant which is transformable to S_1 and reacts well on sugar, we have encountered another (non-transformable) which scarcely attacks it at all. On the other hand, we have not succeeded in getting a change in a bacterium active on sucrose (R_2) to a bacterium which is inactive (R_1) under the influence of R_1 nucleic acid. Positive results have not been obtained yet. We have also failed in our efforts to obtain the reverse process with the R_2 nucleic acid. It is probable that C_2, like the classic "*Coli mutabile*" and various other bacilli, can give rise to spontaneous mutations involving the capacity to attack the same sugar, particularly sucrose. This was the problem we were studying when, about a year ago, we left the Pasteur Institute to assume the difficult task of putting in order the two Institutes of Biological Chemistry and Bacteriology of the Faculty of Medicine in Strasbourg. This resulted in a temporary interruption of our researches, but we have reason to believe that we may now actively resume our studies on the phenomenon of controlled mutations in colon bacilli, and we shall turn our entire attention again to the examination of the biochemical properties of our colonies.

The remarkable phenomenon of controlled mutation in bacteria presents various problems. This phenomenon must, *occasionally*, play a role in the equilibrium of the saprophytic bacterial flora present in natural media (soil, water, etc.), as well as in the equilibrium of the pathogenic flora appearing in wounds and other foci of infection. But it would be risky indeed actually to attempt to evaluate the extent of this role.

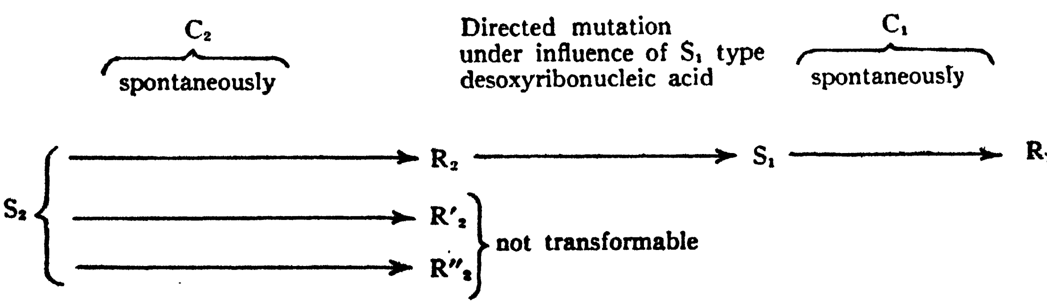


Diagram of directed mutation $C_2 \rightarrow C_1$. (Reverse mutation $C_2 \leftarrow C_1$ has not been possible up to this time.)

On the other hand, phenomena of spontaneous biochemical mutations in bacteria are well known; they can, in certain cases at least, present a reversible character. Phenomena of spontaneous irreversible antigenic mutation (S → R transformation) and phenomena of spontaneous reversible antigenic mutations, manifested by the existence of "phases" involving usually antigen H and sometimes antigen O (see our recent report, Boivin, 1946, on antigen O), are also well known. The question arises as to whether or not there is any relation between the mechanism of these spontaneous mutations and that of the mutations induced and controlled by desoxyribonucleic acid. These are questions which naturally present themselves but to which it does not seem possible to find definite answers in the present state of our knowledge. In fact, we should have these two main points firmly established first:

(1) What is the exact role of desoxyribonucleic acid *in* bacteria?

(2) What is its mode of action *outside* of bacteria, when it induces and directs a mutation?

The two last parts of the present report are devoted to the study of these preliminary questions, which are of great interest for biology in general.

II. Participation of Desoxyribonucleic Acid (and of Ribonucleic Acid) in the Chemical Constitution and Cytological Structure of Bacteria in General, and of Colon Bacilli in Particular

Working with bacteria in our laboratory, Vendrely (Vendrely and Lehoult, 1946; Vendrely, 1946) has applied with certain modifications the method used by Schneider (1945) for the determination of desoxyribonucleic acid and ribonucleic acid in animal tissues. His technique consists essentially of the following steps:

(1) Extraction of bacteria in the cold, using trichloracetic acid, which eliminates an acid-soluble fraction containing, in particular, free purines, purine and pyrimidine nucleotides and nucleosides, and also polysaccharides of the bacteria; this extract is thrown out.

(2) Extraction of bacteria with hot trichloracetic acid, which renders soluble the whole of the two nucleic acids at the cost of partial hydrolysis; this does not interfere with later operations; it is this extract, obtained with heat, to which all determinations refer.

(3) Estimation of the amount of the two nucleic acids by determination of total purines after acid hydrolysis.

(4) Estimation of desoxyribonucleic acid by the Dische color reaction to diphenylamine (very specific).

(5) Estimation of ribonucleic acid by difference, and checking the order of importance of the result found from the Bial-Mejbaum reaction to orcine (slightly specific).

Numerous bacteria have been studied in various physiological states: typhus bacillus and other Salmonella (paratyphus bacillus B and Aertryck bacillus), Shiga bacillus, various colon bacilli, *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Staphylococcus aureus, Bacillus anthracis, Bacillus subtilis* and *Mycobacterium tuberculosis.* Depending on species of bacteria and age of culture, desoxyribonucleic acid represents from 1 or 2 to 5% of dry weight, ribonucleic acid 5, 10, 15, and sometimes even 20% of the same dry weight. Young cultures are much richer than old cultures in ribonucleic acid and slightly richer in desoxyribonucleic acid. No clear correlation could be established between degree of aerobic metabolism and anaerobic metabolism on glucose, ability to multiply *in vitro* under aerobiotic and anaerobiotic conditions, antigenetic structure (smooth form, rough form, etc.) and virulence of the bacteria, on the one hand, and their nucleic-acid content on the other. The various colon bacilli studied (5 in all) have led to somewhat similar results. By way of example we cite the following results for S_1, obtained with bacteria cultured on ordinary agar for 5 hours and for 20 hours at 37°; all are given in percentage of dry weight. (See table below.)

In addition to proteins and nucleic acids, bacterial cells contain, among other things, mineral substances and organic acid-soluble substances, small amounts of lipoid bodies, and particularly polysaccharide material (specific polysaccharides), which amount to about 10% of the dry weight.

Referring to some reports in the literature on animal tissues, one will note that bacteria outclass all organs, with the exception of the spleen and especially the thymus, in abundance of desoxyribonucleic acid, and all organs, with the exception, perhaps, of the pancreas, in content of ribonucleic acid.

In the case of cells of higher organisms, both animal and plant, it is now known that desoxy-

	Total N (determined)	Total Proteins (calculated)	Total Nucleic acid (determined)	Desoxyribonucleic acid (determined)	Ribonucleic acid (determined)
5 hours:	14.2	62.0	21.4	6.1	15.3
20 hours:	14.4	69.1	13.1	4.4	8.7

ribonucleic acid is confined to the nucleus, while ribonucleic acid exists almost exclusively in the cytoplasm. Is the same true of bacteria, and may we assume in them the existence of a nucleus?

After many controversies, there can be no actual doubt of the presence of a true nucleus in bacteria. Applying ultraviolet microphotography, the "nucleal" reaction of Feulgen, and various staining methods, Badian, Neumann, Stille, Piekarski, Delaporte, Knaysi, Peshkov, Robinow, and others, have demonstrated a small organelle appearing to have the morphological value of a nucleus. It was Robinow (1942, 1944, 1945) who obtained the best pictures, working with different bacilli, particularly colon bacilli. His technique consists principally of the following: fixation of bacteria histologically; treatment with N HCl at 60° for several minutes; Giemsa staining. Relatively large, approximately round nuclei, showing divisional figures, can be seen. The short bacillary elements contain a single nucleus; the elongated elements contain several; in sporulated forms the nucleus is found in the spore. These facts have been confirmed in our laboratory by Tulasne (1947) who also obtained fine pictures of the colon bacillus nucleus by the hydrochloric-acid technique. Vendrely and Lipardy (1946) disclosed the chemical mechanism of Robinow's technique. Hydrochloric acid eliminates ribonucleic acid rapidly and desoxyribonucleic acid slowly, so that careful treatment with this reagent suppresses the basophilia of the cytoplasm, leaving almost intact the basophilia of the nucleus.

Quite recently, Tulasne and Vendrely (1947) have just furnished definite proof of the cytoplasmic localization of ribonucleic acid and of the nuclear localization of desoxyribonucleic acid in bacteria, and especially in colon bacilli, using enzymes, ribonuclease and desoxyribonuclease, isolated from beef pancreas and carefully purified. Ribonuclease suppresses cytoplasmic basophilia, leaving nuclear basophilia intact; the latter can be abolished, in turn, by the subsequent action of desoxyribonuclease; finally, by applying desoxyribonuclease alone, one destroys the basophilia of the nucleus without suppressing that of the cytoplasm (see Figs. 1, 2, 3 and 4). Treatment with ribonuclease before Giemsa staining but after proper fixation gives easy and sure evidence of a bacterial nucleus. By this method Tulasne and Vendrely have seen the nucleus of the colon bacillus and of related bacteria (Salmonella, etc.), of *Bacillus anthracis, Corynebacterium diphtheriae,* and *Neisseria gonorrhoeae.*

In the course of autolysis of bacteria there takes place a more or less abundant liberation of nucleic acids, which can be separated from the surrounding medium by acid precipitation of the nucleoprotein matter in which they are found. It is interesting to note that, with colon bacillus, the proportion of desoxyribonucleic acid to total nucleic acid is much higher in the nucleoproteins of autolysed bacteria than in total intact bacteria; it is always higher than 0.50 and often approaches 0.75 or 0.80 in the first case, while it is around 0.20 to 0.30 in the second case (Vendrely, 1947). This fact may be at least partly explained by the existence of a very active ribonuclease in colon bacilli; it is possible to isolate it from the bacteria themselves as well as from their autolysates, and we are actually at work in our laboratory with its purification and with the study of its properties. On the other hand, the same bacteria seem to be lacking in desoxyribonuclease. These observations explain the fact that spontaneous or induced autolysis of colon bacillus yields desoxyribonucleic acid of nuclear origin sufficiently unaltered to be still capable of inducing directed mutations.

Thus, one conclusion is clear: desoxyribonucleic acid of the colon bacillus is confined within a nucleus resembling that of higher organisms, and it can be liberated, without profound alteration, in the course of autolysis. We must now ask ourselves what role is assigned to this acid *in* the bacterial cell, and through what mechanism it becomes capable of inducing directed mutations once it has left the cell. Incidentally, and for the sake of comparison, we shall seek to discover the function of the bacterial ribonucleic acid. The analogy of chemical constitution and cytological structure which is observable between bacteria and other cells entitles us, until more ample information has been obtained, to apply to all cells the conclusions reached concerning the role played in bacteria by nucleic acids and, particularly, by desoxyribonucleic acid.

III. The Role of Desoxyribonucleic Acid (and also of Ribonucleic Acid) in the Life of the Bacterial Cell and the Mechanism of Directed Mutations; its Importance in the General Biochemistry of Heredity

Let us review briefly the ideas generally accepted at this time regarding the role of the two nucleic acids in the cells of higher organisms, animal and plant.

Desoxyribonucleic acid is present in the chromosomes and the genes of the nucleus. Each gene, more particularly, each carrier of one of the hereditary characters of the species, is a special macromolecule of desoxyribonucleoprotein, owing its specificity to its protein component, and not to its nucleic component which is identical for all genes and all species. As for ribonucleic acid, it is present in the cytoplasm—epecially in the microsomes of Claude—in quantities proportional to the extent to which the latter is the site of syntheses, and particularly of more active protein syntheses (Brachet, Caspersson). Let us now see to what extent these ideas can be applied to bacteria.

The existence of genes, and even of numerous

genes, in bacteria can scarcely be doubted. When a colony of colon bacilli is X-rayed (Gray and Tatum, 1944; Tatum, 1945), one often observes the appearance of mutants presenting biochemical requirements unknown in the original colony, i.e., the need to find preformed, in the environment, certain "growth factors" which the microorganism is now incapable of synthesizing. Comparable observations have been made elsewhere by Roepke, Libby, and Small on irradiated colon bacilli, and by Anderson on colon bacilli exposed to the action of phages. This is a situation which exactly recalls that encountered by several authors (Beadle and Tatum in particular) in the course of irradiation of molds. Now, in sexual organisms, whether they be molds in which one studies synthesizing capacity, higher plants in which one studies flower color, or insects in which one studies eye pigmentation, geneticists have reason to believe that every biochemical character depends upon the intervention of one particular gene, a gene which conditions the formation of an enzyme or of a group of enzymes responsible for the appearance of this character. Similarly, one must admit that the same holds true for bacteria, organisms for which *microscopic observation* has not yet permitted irrefutable evidence of some sort of conjugation. It must be added that recent suggestive statements of Lederberg and Tatum (1946) nevertheless favor the existence of at least occasional sexuality in colon bacilli. In fact, these authors have seen that two irradiation mutants with different biochemical deficiencies can, if propagated together, reproduce the original form, without deficiency, and also new forms which combine, in various ways, certain deficiencies belonging to the two strains under experiment. Nothing like it can be obtained when one of the living strains is subjected to the action of an extract of the other strain, which excludes the intervention of directed mutation processes. Therefore we are obliged to admit, with Lederberg and Tatum, the operation of a sexual fusion of bacteria of different genotypes, distributing their genes in new combinations.

As for the exceptional abundance of ribonucleic acid in the bacterial cytoplasm, this is certainly in keeping with the colossal power of reproduction of these organisms, which is in turn linked with their great capacity to produce proteins.

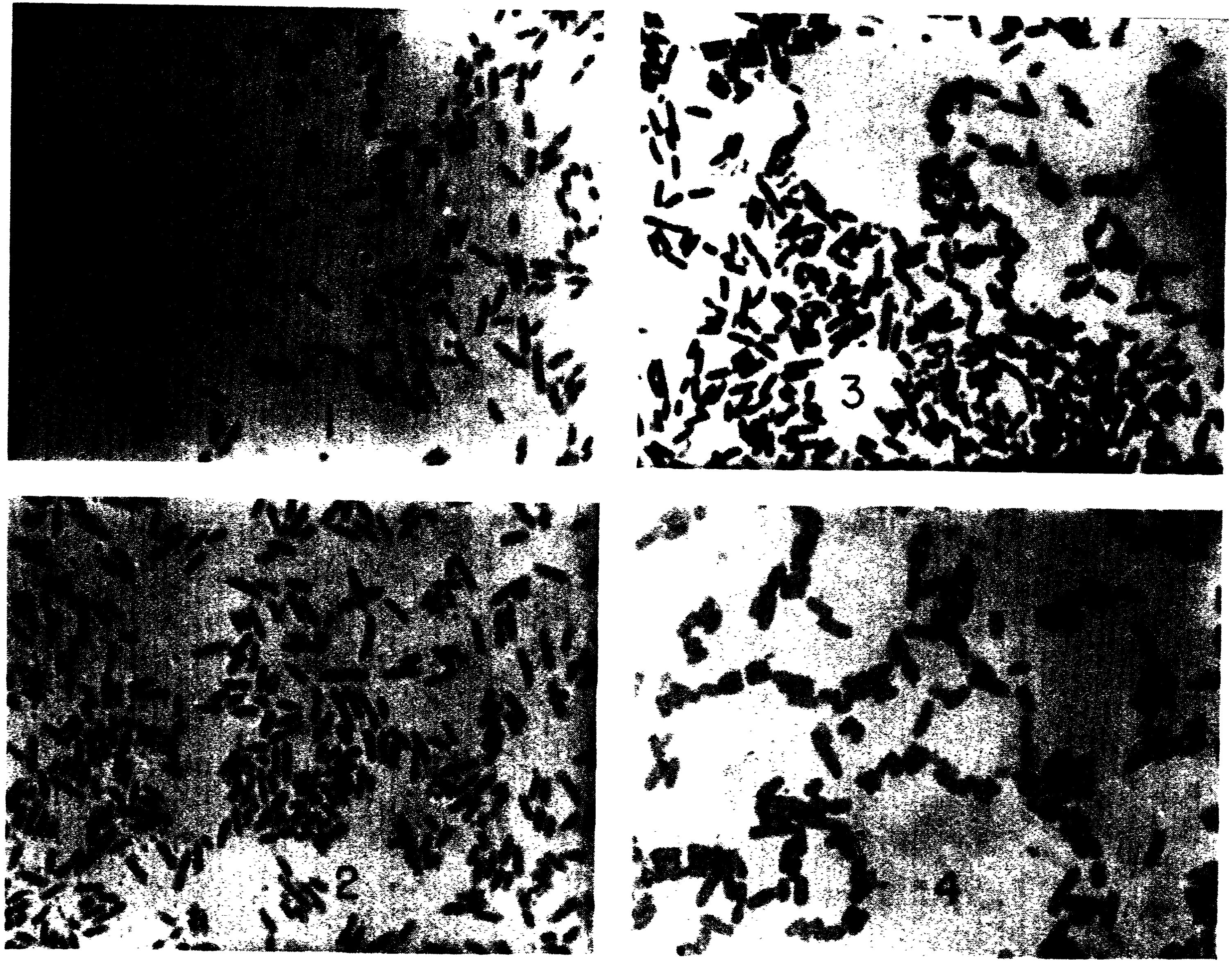
Thus there exist in the bacterial nucleus, as in the cell nucleus of higher organisms, desoxyribonucleoprotein genes which serve as a substratum for the characters of the species.[8] It follows that whatever happens in the phenomenon of directed mutation can hardly be interpreted otherwise than as a result of solution of the bacterial chromosome apparatus without total destruction of its functional value. Now, we have seen that the active principle in directed mutations apparently is not of a nucleoprotein but only of a nucleic nature. Several important consequences and problems arise from this fact, questions and problems which Avery and his collaborators stressed in their first publications.

In bacteria—and, in all likelihood, in higher organisms as well—each gene has as its specific constituent not a protein but a particular desoxyribonucleic acid which, at least under certain conditions (directed mutations of bacteria), is capable of functioning *alone* as the carrier of hereditary character; therefore, in the last analysis, each gene can be traced back to a macromolecule of a special desoxyribonucleic acid.

Concerning directed mutations in bacteria, we have already recognized the necessity of postulating the existence of many desoxyribonucleic acids, differing one from the other. The general conception of genic constitution which we have just formulated increases this necessity. This is a point of view which, in respect to the actual state of biochemistry, appears to be frankly revolutionary. In fact, in view of the analogy with what takes place in proteins, it leads us to consider the possibility of a structure susceptible of differentiating the various desoxyribonucleic acids: a "primary" structure linked with the nature and mode of grouping of nucleotides in the polynucleotide chain, and, eventually, interpolation into this chain of some foreign elements; a "secondary" structure, originating from a state of coiling or clustering of this chain. The explanation is in the hands of the biochemists and biophysicists. In particular, X-ray and electron microscope examination of desoxyribonucleic acids undepolymerized, may reveal a great deal. The hypothesis of the intervention of a secondary structure is particularly stimulating: we know that in the realm of proteins there is more and more tendency to explain, with Pauling, the existence of an infinite variety of antibodies by modifications of form in the polypeptide chain of the globulin of which they are composed.

In what manner is the desoxyribonucleic acid which constitutes a gene, when it is in place within the cell, capable of controlling a particular character of the living organism—of governing, as it were, the production of a certain enzyme or of a certain group of enzymes? In what manner can the same desoxyribonucleic acid, isolated from the bacterium, act, at least in some favorable cases, upon another bacterium, closely related to the first, and penetrate into its interior (as does the phage?) to induce a mutation and to direct it? These are questions whose far-reaching importance for the general biochemistry of heredity cannot be overestimated, but which must remain actually without precise answers for fear of leaving the realm of probability and entering that of pure invention. Whatever the mechanisms in question may be, it

[8] According to Lea (1947), 250 genes are present in the cell of the colon bacillus.



Figs. 1, 2, 3 and 4 (Boivin). See reverse side for legend.

FIGS. 1 and 2. (Two views of the same preparation). Colon bacillus (× 2000).

Culture of 4 hrs. at 37°.

Fixation with Chabaud mixture (alcohol, formole, phenole, acetic acid).

Action of ribonuclease (10 min. at 60°).

Giemsa staining.

Only nuclear basophilia persists. Note the numerous figures of nuclear division.

FIG. 3.

Same colon bacillus, same fixation.

Direct Giemsa staining: total basophilia.

FIG. 4.

Same colon bacillus, same fixation.

Action of ribonuclease (10 min. at 60°), then of desoxyribonuclease (2 hrs. at 37°).

Giemsa staining.

All basophilia has disappeared.

NOTE: The swelling of the bacteria which is produced on the preparations treated by the enzymes, is an "artefact" largely due, it appears, to the prolonged contact of the bacilli with the hot aqueous liquids.

Preparations and photographs by R. Tulasne and R. Vendrely

is very likely that an experiment will be attempted, sooner or later, for the purpose of introducing into the cell of a higher organism, with the aid of a micromanipulator, some desoxyribonucleic acid from an organism of a related species, isolated by as delicate a method as possible—for example, by that of Mirsky and Pollister. What will be the result? This is the secret of the future. But at the present moment it seems certain that, in the years to come, the minute bacteria will play a primary role in genetic laboratories and compete for first place with the now famous Drosophila.

When we consider the close relationship in chemical constitution of the two nucleic acids, it becomes logical (by analogy and until contrary proof has been furnished) to admit the existence of ribonucleic acids which differ from one another, and to attribute to them functions within the cellular cytoplasm resembling those ascribed to the desoxyribonucleic acids of the nucleus. This led us to formulate more than a year ago (in a Conference of the Société de Biologie d'Alger, April, 1946), the hypothesis that the various cytoplasmic ribonucleic acids are the carriers of acquired characters in microorganisms (enzymatic adaptation according to Karström), and in metazoa the carriers of characters specific to each cellular type (cellular differentiation); they perform these functions by governing directly the enzymatic equipment of the cytoplasm (Boivin and collaborators, 1946 and 1947). This hypothesis would permit a precise statement, at the same time, of both the ideas of Claude and Brachet on microsomes, and those of Wright and Darlington on plasmagenes. It would easily accord with the observations of Spiegelman, Lindegren, and Lindegren (1945), which demonstrate the possibility that an enzyme may multiply autocatalytically, even in the absence of the corresponding gene as well as with the findings of Spiegelman and Kamen (1946) concerning the role of nucleic acid in the synthesis of proteins and enzymes. Finally, it finds excellent support in the fact that, according to Spiegelman (1946), one could *specifically* accelerate the enzymatic adaptation of a yeast to a sugar by means of a nucleoprotein fraction (apparently with ribonucleic acid base, according to his mode of preparation) drawn from the same yeast previously adapted to this sugar. It remains to be determined how ribonucleic acids can control the enzymatic equipment of the cytoplasm, while they themselves are subjected to control by the desoxyribonucleic acids of the nucleus. But here again it does not seem possible actually to launch out into so vague a theory. We may, at the most, catch a glimpse of a series of catalytic actions, which set out from primary directing centers (the desoxyribonucleic genes), proceed through secondary directing centers (the ribonucleic microsomes-plasmagenes), and thence though tertiary directing centers (the enzymes), to determine finally the nature of the metabolic chains involved, and to condition, by this very means, all the characters of the cell in consideration. Ultimately, it remains to be contermined by what mechanism the molecules of ribonucleic acids and those of desoxyribonucleic acids can "multiply," by some autocatalytic duplication, the former in the cytoplasm, the latter in the nucleus, during cellular proliferation.

Thus, this amazing fact of the organization of an infinite variety of cellular types and living species is reduced, *in the last analysis*, to innumerable modifications within the molecular structure of one single fundamental chemical substance, nucleic acid, substratum of hereditary as well as acquired characters. This is the "working hypothesis" quite logically suggested by our actual knowledge of the remarkable phenomenon of directed mutations in bacteria.

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Discussion

Weil: (Dr. Weil discussed experiments on type transformation. Induced type transformation of *Shigella paradysenteriae* (Flexner) has been obtained in 3 out of 225 experiments. The inducing principle is contained in filtrates of broth cultures. Details of these experiments and results were published in a paper: A. J. Weil, Experimental type transformation of *Shigella paradysenteriae* (Flexner), Proc. Soc. exp. Biol. Med. *64*, 1947).

The data available at present do not permit us to make any statements concerning the "specificity" range of the inducing factors. That is, we do not know whether or not the transforming effect is restricted to closely related bacteria; for instance, whether the factor from a colon bacillus may induce transformations in colon bacilli only or whether it may influence other Enterobacteriaceae or maybe even more distantly related microorganisms. As induced transformation has as yet been successful only in rare instances within the range of the same species, this point could be ascertained only by a very large number of experiments. If this is not clearly understood, Dr. Boivin's mentioning of specificity controls may conceivably prejudice the scope of our approach. It is in this respect pertinent to point out that, though bacteriologists freely employ terms like genus and species, very little is known about their exact meaning in microbiology.

Boivin: I have been extremely interested in the results obtained by Dr. Weil. Like him, I have always had the greatest difficulty in obtaining the R form of the Flexner bacillus; neither simple aging of the cultures, nor culturing in the presence of the antisera has been effective, while, under the same conditions, the Shiga bacillus and most of the colon bacilli pass readily from the S form to the R form. In the group as a whole, the Salmonella occupy an intermediate position; they give R variants, but much less readily than most of the colon bacilli. We have no explanation for these facts. It should be emphasized that, when first isolated from their natural medium, the intestinal contents, colon bacilli are always in their S form; the R form is thus a kind of artefact, appearing only under the artificial conditions of laboratory culture media.

Among the pneumococci and colon bacilli, the

transformation of antigenic type, through the inducing effect of a desoxyribonucleic acid, takes place by passage of the R form of one type into the S form of another type. The great difference in appearance of the cultures in broth and of the colonies on agar, between the R and S forms makes recognition of the transformation possible before serological tests are made. Dr. Weil has not had this advantage in the work with the Flexner bacillus. Furthermore, it is quite possible that the usual absence of the R form in the Flexner bacillus makes the passage from one antigenic type to another much more difficult. These reasons will explain the small percentage of transformations obtained by Dr. Weil. But on a scale of this size, positive experiments which have been carefully performed have a decisive value even though the frequency with which they can be repeated remains low. As I have said earlier, it may well be that certain inherent factors in the cultures—natural factors still entirely unknown—take part, as far as the number of bacteria to undergo transformation and the concentration of desoxyribonucleic acid which may be effective are concerned, in determining the frequency of transformation of antigenic type in colon bacilli. In pneumococci, Avery and his colleagues have already been able to show the importance of different factors in blood serum or in ascitic fluid (McCarty, Taylor and Avery, Cold Spring Harbor Symposium Quant. Biol. *11*:177, 1946).

Dr. Weil raises the question of an eventual transformation of colon bacillus into Flexner bacillus or vice versa. This does not seem fantastic to me. The Salmonellas, the dysenteriae and the colon bacilli are grouped together within the immense group of intestinal bacteria, having in common many morphological, biochemical and even antigenic characters. One knows how frequently in colon bacilli are found antigenic factors characteristic of dysenteriae or Salmonellas; even the Vi antigen of the virulent typhus bacillus (Felix, Kauffmann) has been found in several bacteria which are unhesitatingly classified as colon bacilli by the details of their biochemical properties and by their lack of virulence. The close family relationship existing between the different intestinal bacteria permits one to hope for the possibility of extended antigenic and biochemical transformations in bacteria by the process of directed mutations. Experience will decide. If one is able to demonstrate the reality of such a process, one will reorganize certain aspects of epidemiology, thus far based upon the dogma of the absolute separation and stability of the species of pathogenic bacteria, even neighboring species. As for the possibility of the passage by directed mutation of one bacterium into another which differs greatly in general characters (for example, pneumococcus ⇄ colon bacillus) it would seem *a priori* to be highly improbable. But again, experience will be the judge.

BRACHET: I am surprised at your finding that autolysates from the bacteria are active in your experiments. Couldn't one expect these autolysates to contain desoxyribonuclease which would depolymerize the high molecular DNA? And, if so, wouldn't these results suggest the possibility that other substances than DNA are also active?

BOIVIN: Colon bacilli contain a very active ribonuclease, which is recovered in their autolysates. Our attention is now directed to purifying this enzyme and studying its properties; it seems to be less thermostabile than pancreas ribonuclease. It can be substituted for pancreas ribonuclease in the demonstration of bacterial nuclei. On the other hand, colon bacilli seem to contain only very minute amounts of desoxyribonuclease. This explains how the autolysates contain more desoxyribonucleic than ribonucleic acid, and how the former keeps its power of inducing transformation of antigenic types (conservation of an adequate state of polymerization). Further, one can guard against all action of a bacterial desoxyribonuclease by performing the autolysis in the presence of citrate. McCarty (J. gen. Physiol. *29*:123, 1946) has shown that desoxyribonuclease requires for its activity the presence of a metal such as magnesium, whose effect is inhibited by citric acid. We have confirmed this fact, and in addition have noted that the magnesium is not required by ribonuclease, whose action is not stopped by citric acid.

It is now known that in bacteria, as in higher organisms, various chemical substances can increase the frequency of spontaneous mutation. The possibility is by no means excluded that agents of this kind are elaborated by certain bacteria and may be recovered in bacterial autolysates and in culture filtrates. Perhaps they will show themselves to be capable of increasing the frequency of directed mutations induced by specific desoxyribonucleic acids. An immense field of research opens to the activity of investigators, that of bacterial interactions other than antibiotic effects. It cannot fail to offer interesting results on the double plane of general biology on the one hand, and of bacterial ecology and epidemiology on the other.

MIRSKY: The work on transformation of bacterial types has been a healthy stimulus to the chemical investigation of nucleic acids. Discussions at this symposium have clearly shown that these wonderful bacteriological discoveries have caused chemists to consider critically the evidence for uniformity among nucleic acids, and the generally accepted conclusion is that the available chemical evidence does not permit us to suppose that nucleic acids do not vary. At present, however, the evidence for specificity of nucleic acids comes entirely from bacteriological experiments. Implications concerning the specificity of nucleic acids are, as Dr. Boivin has said, revolutionary for the chemical aspects of biology. It is, accordingly, necessary to consider

just how cogent the evidence is for the specificity of nucleic acids.

The evidence for specificity may be briefly summarized.

1) The material effective in causing transformation consists of a highly polymerized desoxypentose nucleic acid. This nucleic acid is as pure as is any that has been prepared from the thymus gland and other sources, but only a nucleic acid prepared from the proper type of bacterial cell is effective as a transforming agent. No protein can be detected in a purified preparation.

2) The active principle is not destroyed by proteolytic enzymes.

3) Only an exceedingly small quantity of the apparently specific nucleic acid is required.

4) The active principle is inactivated by desoxyribonuclease and at the same time the nucleic acid is depolymerized.

The significance of each point will be considered.

1) The criteria of purity for nucleic acids are at present so inadequate that 2 to 3% of protein may be present in a so-called pure preparation. With a sensitive protein test like the Millon reaction, for example, this much protein in a sample of nucleic acid would not be detected. Lack of antigenicity in a nucleic acid preparation is an unreliable indication that no protein is present because all of the proteins present in chromosomes (and these are the proteins of the cell that are known to be associated with desoxypentose nucleic acid) have failed, so far, to act as antigens. The best available criterion for the purity of a nucleic acid is the nitrogen-phosphorus ratio, and this cannot be used to exclude the presence of several percents of protein.

2) Many native proteins, the tobacco mosaic virus protein, for example, are not acted upon by proteases until they have been denatured.

3) The quantity of material that suffices to produce transformation appears to be exceedingly small when expressed gravimetrically, but when the number of particles required is calculated, it is found that the number is of the order of 100,000,000 or even more. It should be recalled that only one phage particle is required to produce lysis in a suspension of bacteria. It is difficult to decide whether the quantity of transforming agent required should be considered to be large or small. If 2% of protein were present, the number of protein particles in the material used for transformation would not seem to be unduly small.

4) The action of desoxyribonuclease shows that the activity of a preparation depends upon the presence of polymerized desoxyribonucleic acid. This does not, however, permit us to conclude that the specificity of the active principle is a nucleic acid specificity. The action of this enzyme is satisfactory, though certainly not conclusive, evidence that the active principle is either desoxyribonucleic acid itself or a desoxyribonucleoprotein. If we choose the former alternative, it follows of course that the specificity of the active principle is due to nucleic acid; if we choose the latter alternative, it may imply that specificity resides in the protein moiety of a nucleoprotein. And, as we have seen, nucleoprotein may well be present in the highly purified active principle.

In the present state of knowledge it would be going beyond the experimental facts to assert that the specific agent in transforming bacterial types is a desoxyribonucleic acid. It should also be stated that there is no need to believe that the specificity of the active principle must depend upon the presence in it of a specific protein. Discussions at these meetings have brought forward suggestions for experiments that may perhaps decide whether the transforming agent is a nucleic acid or a nucleoprotein.

Boivin: It is true that one cannot say with absolute certainty that it is the action of desoxyribonucleic acid and not of some trace of active protein which takes part in directed mutation, in the case of pneumococci as well as colon bacilli. But the intervention of desoxyribonucleic acid is extremely likely, and it seems to us that the burden of the proof rests upon those who would postulate the existence of an active protein lodged in an inactive nucleic acid.

As one successively, by chemical fractionation, further increases the activity of the preparations responsible for type transformations, one further approaches pure desoxyribonucleic acid. In this direction, Avery has gone further than we have, and has arrived at a desoxyribonucleic acid as pure as it is possible actually to obtain.

The activity resists the action of ribonuclease, and it is very rapidly abolished by desoxyribonuclease (prepared by the method of Fischer or of McCarty).

The activity resists the action of proteinases (pepsin, trypsin, papain).

When one examines a nucleoprotein from autolysis of colon bacilli, this proves to be strongly antigenic in the rabbit. After a short pepsin digestion, it has lost all antigenic power and has become incapable of precipitation by the immune serum. Similarly, the nucleic preparations resulting from fractionation of the product of pepsin digestion by HCl at low temperature are not antigenic and are not precipitated at all by the antibody which interacts with the nucleoprotein. In spite of this, they are highly capable of inducing transformations.

The hypothetical active protein would have to be non-antigenic and resistant to proteolytic enzymes; it would have to be capable of extraordinary biological activity; finally, most difficult to envision, its activity would have to be abolished when, under the effect of the desoxyribonuclease, one had depoly-

merized the nucleic *impurity* which resembles it.

The rule of economy in hypotheses inclines one to think, until proof to the contrary is presented, that it is actually desoxyribonucleic acid which is the agent under consideration.

TAYLOR, H.: The group of us working on pneumococcus transformation was most excited by Professor Boivin's reports on transformation in *E. coli.* It was particularly interesting to see the parallelisms between the two experimental systems. Professor Boivin has spoken of the existence of several types of R_2 cells in his R_2 cultures, only one of which types is capable of being transformed. He has drawn attention to a similarity in the R strain of pneumococcus. I should like to emphasize one thing about the variability of the R cells, since it is an important aspect of the transformation story. Although several kinds of variants do arise in the R culture, none of them is capable of synthesizing type specific polysaccharide. Actually the R strain is quite stable, the appearance of variant types being an unpredictable thing, probably dependent on the variable selection factors operating in the complex culture medium. There is no reason to believe that the transformable R strain of pneumococcus is an especially variable organism.

BOIVIN: As C_2 passes spontaneously from the form S to the form R, one obtains a whole spectrum of variants: R_2, R'_2, R''_2, R'''_2, etc., differentiated from each other by the appearance of their colonies. Only one of these, R_2, is shown to be capable of transformation into S_1 through the action of the desoxyribonucleic acid of S_1. This has not led us to characterize R_2 as "unstable." But I would like to state clearly that it is a question of a potential and contingent instability, not definitely showing itself except in the presence of the desoxyribonucleic acid of S_1. Left to itself, R_2 is as stable as R'_2, R''_2, R'''_2, etc., and does not give rise to any spontaneous transformation. To avoid all confusion, it is better to abandon the qualification "unstable" and to say, simply stating the facts, that R_2 is "transformable" while R'_2, R''_2, etc. are not, or at least are not transformable with a sufficient frequency to be readily revealed by experiment. From this point of view, as from all the others, the colon bacillus behaves exactly like the pneumococcus.

ADDITIONAL COMMUNICATION BY A. BOIVIN

Boivin, A., Vendrely, R., and Tulasne, R. (Strasbourg): In the demonstration of the bacterial nucleus, the method of ribonuclease shows itself to the more general than the HCl method of Robinow. It has made possible the demonstration not only of the nucleus of colon bacilli and related bacteria (Salmonella, *dysenteriae,* etc.) but also of *Bacillus anthracis* and allied bacteria (*B. subtilis,* etc.), that of *Corynebacterium diphtheriae* and that of *Neisseria gonorrhoeae. C. diphtheriae* (thus almost depleted of metachromatic granules) shows in young cultures a remarkable cytological polymorphism: at times it takes the form of a bacillus possessing a single large oval nucleus, and at other times of a bacillus containing a row of small rounded nuclei. *N. gonorrhoeae* in culture as well as in its intracellular milieu (in urethral pus) shows a rounded nucleus in very clear cytoplasm.

Staphylococcus aureus resists Robinow's method as well as the ribonuclease method. But after treatment of the bacteria with lithium carbonate in saturated aqueous solution, ribonuclease makes possible the demonstration of a single rounded nucleus, or often of two more or less enclosed nuclei, present in each coccus. It is probable that the effect of the lithium carbonate is to decrease the solidity of the combination between ribonucleic acid and the bacterial proteins, more or less denatured by the reagent, and thus to make the ribonucleic acid accessible to the action of the ribonuclease. In every case, it can be verified that the basophilic organelle left by the ribonuclease is Feulgen positive, and that after the action of the desoxyribonuclease it loses all basophilia. It is thus a question of a desoxyribonucleoproteic structure. In addition, its very appearance and the numerous division figures which it shows in young bacteria lead one without hesitation to give it the status of a nucleus.

The streptococcus, the pneumococcus and the Koch bacillus are being studied at present, but have so far given only unclear pictures.

THE METABOLISM OF NUCLEIC ACIDS DURING EMBRYONIC DEVELOPMENT

JEAN BRACHET

Thanks mainly to the development in recent years of techniques for the cytochemical detection of both types of nucleic acids (staining with basic dyes and digestion with nucleases, ultraviolet spectroscopy), considerable progress has been made in our understanding of the metabolism of nucleic acids during embryonic development. Two main problems will be considered here; the mechanism of nucleic acid synthesis during embryogenesis, and the possible role of nucleoproteins in embryogenesis.

Nucleic Acid Synthesis During Embryonic Development

It was shown long ago that the type and the magnitude of nucleic acid synthesis varies in different species. In the hen's egg there is very little nucleic acid present before development and a considerable synthesis of both desoxyribonucleic and ribonucleic acids occurs in the embryo (Plimmer and Scott, 1909; Calvery, 1929, etc.). In the sea-urchin egg, on the contrary, as found by Masing and confirmed by the later work of J. and D. Needham, the unfertilized egg contains a very large amount of nucleoprotein phosphorus and purine bases; the pluteus larva, although it contains hundreds of nuclei, shows little or no synthesis of these nucleic acid constituents. Until 1930, the explanation given for this remarkable finding was that the unfertilized egg probably contains a cytoplasmic reserve of desoxyribonucleic acid, which at that time was still believed to be the only nucleic acid present in animal tissues. Such an explanation, however, had to be given up when it was found by various investigators that the Feulgen reaction, characteristic of desoxyribonucleic acid, is always negative in the cytoplasm of normal cells, including oocytes and developing eggs. A study of the sea-urchin egg (Brachet, 1933), using the Feulgen method, showed that there was no exception, in this case, to this rule. The unfertilized and just fertilized eggs give only a very faint positive test in the nucleus, and no reaction at all in the cytoplasm. Later stages of development (blastulae, gastrulae and plutei) stain, on the contrary, more and more intensely as the number of nuclei steadily increases. The cytochemical evidence thus suggested that desoxyribonucleic acid synthesis, occurring in the nuclei, is a constant feature of embryonic development.

There was only one possibility left to explain the apparent contradiction of desoxyribonucleic acid undergoing a considerable synthesis during development while the total nucleoprotein phosphorus and purine content remained constant; that was to assume that the unfertilized egg contains a reserve of the Feulgen negative ribonucleic acid and that this acid is converted into desoxyribonucleic acid. This hypothesis, when I first made it in 1931, was rather revolutionary, in view of the fact that it was commonly believed then that ribonucleic acids were to be found only in plant cells and in the pancreas. However, estimates of the pentose and of the desoxyribonucleic acid content of the sea-urchin egg at various stages (Brachet, 1933, 1937) showed that during development there is a steady decrease in pentoses and an equivalent synthesis of desoxyribonucleic acid. Although the agreement between desoxyribonucleic acid synthesis and the pentose decrease was very satisfactory, it seems necessary to recheck the results, now that better and more modern microanalytical methods have been developed, because the sea urchin egg is the only case where *quantitative* evidence for a conversion of ribonucleic acid into desoxyribonucleic acid has been found. As such a conversion is now often supposed to take place in many types of cells, it is quite important that the case of this conversion in the sea-urchin egg rests on an absolutely firm basis. Such a reinvestigation of nucleic acid synthesis in the sea-urchin egg is planned for the near future.

It is, however, highly probable that conversion of ribonucleic acid into desoxyribonucleic acid can take place in many cells at the time of mitosis. The cytoplasm of dividing cells is usually less basophilic than that of neighboring resting cells (Brachet, 1942, and unpublished observations). X-radiation which, of course, blocks mitosis, produces an accumulation of ultraviolet-absorbing material (ribonucleic acid) in the cytoplasm (Mitchell, 1942) while it simultaneously stops desoxyribonucleic acid synthesis (Stowell, 1945). According to Mitchell, X-radiation probably inhibits the conversion of ribonucleotides into desoxyribonucleic acid.

Cytochemical observation, using basic dyes and ribonuclease, made on various eggs, further strengthens the hypothesis that ribonucleic acid is converted into desoxyribonucleic acid. The cytoplasmic basophilia due to ribonucleic acid, in the sea-urchin egg, steadily decreases during development. Cytochemical observation thus agrees quite well with quantitative findings (Brachet, 1945). According to a personal communication from Professor J. Pasteels, one of his co-workers at the University of Brussels, M. Mulnard, recently made cytochemical observations on insect eggs which agree

perfectly with the hypothesis of the conversion of ribonucleic acid into desoxyribonucleic acid. In these eggs, ribonucleic acid is present in the cortical cytoplasmic layer, where it shows a cephalo-caudal gradient, and in granules scattered in the centrally located yolk. The nuclei in this central region cleave as usual, but the divisions come to a standstill when the stock of basophilic granules is exhausted. The ribonucleic acid in these granules is presumably utilized for the building up of the nuclei containing desoxyribonucleic acid. These latter then migrate into the peripheral cytoplasmic layer, where they resume their mitotic activity, which is greatest in those regions where the ribonucleic acid content is highest.

Let us now return to the case of the chick embryo, where there is a synthesis of both ribo- and desoxyribonucleic acid during development, and try to find out why the behavior here is so different from that observed in the sea-urchin egg. The most obvious difference between these two types of eggs is that the amount of growth and morphogenesis is much greater in the complicated chicken embryo than in the simple, primitive sea-urchin egg. In particular, there is a tremendous utilization of reserve yolk, and a considerable building up of new proteins during the development of the chick. Now, it is known from the cytochemical work of Caspersson (1941) and of Brachet (1941) that there is always a marked accumulation of ribonucleic acid in any type of cell where active protein synthesis is proceeding, whether they are enzyme-secreting cells, such as those of the pancreas or of the gastric mucosa, growing cells, regenerating cells, neurones, maturing oocytes, or the silk-secreting cells of the silkworm. It is thus to be expected that when the chick embryo is growing, its cells will be the site of a synthesis of nucleic acids which will slow down whenever growth and differentiation stop in any particular organ. Such an expectation is in accordance with Schaeffer and LeBreton's finding that the ratio: $\frac{\text{Purine nitrogen}}{\text{Total nitrogen}}$ begins to decrease when growth of the embryo slows down. Furthermore, cytochemical observations by Brachet (1940), and by Caspersson and Thorell (1941) show conclusively that both in the chicken and in the Amphibian embryo, the ribonucleic acid content of each individual organ increases when it begins to grow actively. Later on, both basophilia and ultraviolet absorption decrease, at the time when growth becomes slower and cytological differentiation is under way. But, if one observes cells of the young, actively growing embryo, *which are in the actual process of division,* it is found that their cytoplasm is less basophilic than that of neighboring resting cells (Brachet, 1946).

The metabolism of nucleic acids during development may thus be integrated without difficulty into our general knowledge of nucleic acid metabolism in other cells. The ribonucleic acid content of cells will vary in *opposite* directions in different circumstances. It will increase whenever there is a synthesis of new proteins, as a result of growth processes or elaboration of specific proteins during organogenesis; but there will be a conversion of ribo- into desoxyribonucleic acid during mitosis itself, especially whenever the cells cleave without an increase in cellular volume, as is the case during segmentation of the egg. While synthetic processes predominate in the chicken embryo, cell division has the upper hand in the sea-urchin egg. It is, of course, to be expected that all intermediate stages are to be found between these two extreme cases, and such an expectation is realized when one analyzes J. and D. Needham's data on nucleoprotein phosphorus synthesis during development in the eggs of various species.

One more point should be emphasized here. If the scheme outlined above is correct, one should expect that, in the eggs of Amphibia, for example, conversion of ribo- into desoxyribonucleic acid occurs during the early stages of development (segmentation and possibly gastrulation), while in the later periods of growth and organogenesis both types of nucleic acids should be simultaneously synthesized. Both the cytochemical and analytical data at hand point towards such a view. The basophilia of the cytoplasm decreases during cleavage, especially at the late blastula stage where the nuclei suddenly become smaller and much richer in desoxyribonucleic acid. This decrease in basophilia is especially obvious in Amphibian hybrids (*Rana esculenta* ♀ × *Rana fusca* ♂), where development is blocked at the early gastrula stage (Brachet, 1945). In later stages, the intensity of both the Feulgen reaction and of the staining by basic dyes markedly increases, following a gradient pattern which will be described below. As regards quantitative measurement, it has been shown by Graff and Barth that the purine content remains constant in the frog egg during cleavage and then increases progressively. Our own observations, part of which were published in 1941, also indicate that ribonucleic acid synthesis between fertilization and mid-gastrulation is only 7%, within the limit of error of the method used, but that this synthesis reaches 23% at the mid-neurula stage, and 62% at hatching. More recent estimates using Schneider's method, were made comparatively on morulae and late blastulae or young gastrulae from the same batch of eggs. It was found that during this period there is, according to what should be expected from the cytochemical observations and Graff and Barth's purine estimations, a 10% to 15% drop in the ribonucleic acid, associated with an equivalent synthesis of desoxyribonucleic acid.

The Localization and Role of Ribonucleoproteins During Morphogenesis

A. *Artificial Parthenogenesis*

It has been known since Bataillon's early work, that simple pricking of a frog egg with a sterile needle produces only an activation reaction, i.e., the cortical changes characteristic of fertilization. True cleavage and eventually later development occur, however, only if a nucleated blood cell is introduced into the egg with the needle. Little is known of the chemical nature of this so-called "second factor" which is present in nucleated cells. The work of Einsele (1930) indicated that it is probably a protein or nucleoprotein, possibly of enzymatic nature. Since that time no more research has been done on this subject until recently, when the matter was reinvestigated by Mr. J. Shaver at the University of Pennsylvania, and some interesting facts have already been found by him. Unfertilized, uterine eggs of *Rana pipiens* have been injected, using micropipettes and a simple injection apparatus, with various cell-free extracts from frog sperm, amphibian and mammalian blood cells, blood serum, unfertilized frog eggs and frog embryos in various stages. The extracts were made in dilute (M/200) phosphate buffer, at pH 7.0-7.5, and three fractions were isolated by differential centrifugation, according to Claude's method: large granules (isolated after centrifugation at 6,000 $\times$ g for 30 minutes), microsomes (isolated after 18,000 $\times$ g for 20 minutes), and the supernatant fluid from the high-speed centrifugation. It was found that the greatest activity is present in the "large granules" from the frog testis extract, which gave up to 79% of partially or totally cleaved blastulae; much less activity was seen in the microsome fraction from testis extract, and the supernatant fluid and the buffer solution injected as a control, were negative. Granules from frog blood cells also proved active, while the supernatant fluid and serum from the blood extracts were negative after thorough high-speed centrifugation. Finally, all the extracts from unfertile frog eggs proved inactive, while the large granules from gastrulae and late blastulae (but not morulae) gave positive results.

The activity of these granules remains intact after heating at 50° C for one hour, but it disappears after heating to 60° C for five minutes. The activity is also destroyed after digestion with ribonuclease (1 mgm./100 cc. for 30 min.), but, as shown by Schneider's work on the succinoxidase activity of large granules, this action of ribonuclease might well be due to proteolytic impurities. It seems unlikely that the active portion of the granules is ribonucleic acid itself, since this acid is present in the granules isolated from the unfertilized frog egg (Brachet and Chantrenne, 1943), which seem inactive as a 'second factor.' Since it is known (Claude, 1946; Moog and Steinbach, 1946; Chantrenne, in press) that the large granules contain many more enzymes than the microsomes, although they are poorer in ribonucleic acid, it is possible that the second factor in parthogenesis will ultimately be identified with one of the enzymes which have been found to be specific constituents of these large particles, like cytochrome oxidase, succinic dehydrogenase (Stern, 1939; Chantrenne, 1943) or adenyl pyrophosphatase (Moog and Steinbach, 1946; Chantrenne, in press). Future work along this line will be awaited with great interest.

B. *Evocation of the Neural Tube*

It is, of course, a well-known fact that the formation of a neural tube can be induced (or, better, 'evocated') by the implantation in a young Amphibian gastrula of various tissues or chemicals. The possibility that nucleic acids or nucleotides may play a part in this phenomenon was first put forward by F. G. Fischer, when he found that thymonucleic acid, muscle adenylic acid and adenosine-triphosphoric acid (ATP) are active evocators. However, other organic acids, such as oleic, are also active. So, too, are various derivatives of the sterols, as J. Needham and Waddington demonstrated; these include the sex hormones and the carcinogenetic hydrocarbons. The sterol fraction of the embryo, however, shows very little activity and it is to be remembered that thorough extraction of the lipids present in killed tissues does not at all reduce their evocating power (Holtfreter, 1946; Barth and Graff, 1938).

This insolubility of the evocating agent in lipid solvents led Barth and Graff to the conclusion that it is probably a protein. However, pure proteins, like ovalbumin or serum protein, are known to be inactive. A powerful fraction was prepared by Barth and Graff by extracting Amphibian embryos with dilute NaOH and precipitating the protein with acetic acid. Extracts made with 1 M NaCl and precipitated by dilution, or protein-precipitated by shaking an aqueous extract with chloroform, proved practically inactive. Such behavior points towards nucleoproteins rather than pure proteins as active evocating agents.

Barth and Graff's results were in good agreement with various observations I made at the same time (Brachet, 1943), following a rather different line. When various killed organs or nucleoproteins are grafted into the blastocoele of early gastrulae, it is found that they usually lose their basophilia. The cytoplasmic basophilia, due to ribonucleic acid, disappears much faster than the nuclear basophilia reflected in the Feulgen test for which desoxyribonucleic acid is responsible. It was also found that a good correlation exists between this loss of basophilia and the positive results of the operation. Only when nucleic acids disappear in the graft during its stay in the host is a neural tube evoked. Furthermore, the host epiblast in contact with the graft

apparently is the site of marked synthesis of ribonucleic acid, as indicated by an increase in cytoplasmic basophilia; this occurs, however, only when the evocation of a neural tube takes place. There is thus an inverse relationship between the basophilia of the graft and that in the epidermis at the end of the experiment. These observations suggested that when nucleoproteins are grafted into a gastrula, the nucleic acid is broken down by the host; and, at least, it was indicated that the graft does not necessarily remain inert in the host. The probability of ribonucleic acid breakdown in the graft by the adjoining cells is further strengthened by the finding that the gastrula contains ribonuclease; the much slower breakdown of desoxyribonucleic acid agrees also with the failure to demonstrate the presence of large amounts of thymonucleodepolymerase.

The next step was to graft into the gastrula nucleoproteins from widely different sources, containing various amounts of ribonucleic acid (Brachet, 1944). These nucleoproteins were prepared by ultracentrifugation of extracts made from amphibian gastrulae, liver, kidney, yeast, barley embryos, etc. The ribonucleic-acid-rich pellets were implanted after fixation with alcohol and several washings with sterile saline solution; in many instances, the proteins from the supernatant were precipitated with alcohol and grafted. These latter fractions have, of course, a low nucleic acid content. Crystalline tobacco mosaic virus, treated with alcohol, was also used as a source of nucleoprotein. In the case of this virus and of the nucleoprotein pellet from amphibian liver, implantations of material digested with ribonuclease were also made. The results of these experiments will be found in Table 1.

As can easily be seen, the evocating activity of the nucleoproteins, regardless of their origin, is directly proportional to the ribonucleic acid content. Treatment with ribonuclease almost completely suppresses the activity and it may be added that extraction with hot water or heating in an oven destroys the evocating activity at the time when the ribonucleic acid has been removed or destroyed. Finally, analytical estimates of the ribonucleic acid content of the fractions studied by Barth and Graff showed the same parallelism between ribonucleic acid concentration and activity. Only the alkaline extract, in contrast to NaCl extracts and the protein-chloroform gel, has a high ribonucleic acid content.

These results clearly indicate that the evocating activity of killed tissues or extracts of these tissues is closely linked to their ribonucleic acid content. The latter substance is broken down when the graft is implanted into the host blastocoele, and it is thus likely that breakdown products of ribonucleic acid (mononucleotides?) are responsible for the evocation.

However, it should be kept in mind that these experiments tell us nothing about the way the normal living organizer is acting, since an entirely different mechanism might be at work. Furthermore, we have now to appraise the value of these results in a more critical way.

A great danger in this type of experiment, lies in the fact that cytolysing cells release a substance or substances which act as powerful evocators. This fact which has been emphasized by Barth and Graff and by Holtfreter, is of considerable importance, since any toxic substance which kills some of the neighboring cells may be expected to act as an evocator. The possibility that ribonucleic acid, or its breakdown products, may act in this indirect way has by no means been excluded. Actually, the very fact that the ribonucleic acid present in the graft is broken down by adjoining cells is quite suspicious and suggests the possibility that some of these cells have undergone cytolysis. With the hope of settling this question, I have very recently cultivated presumptive ectoderm explants in saline solutions containing rather small amounts of nucleic acids or nucleotides (0.5 mgm/cc. of yeast nucleic acid, yeast adenylic acid, guanylic acid, muscle adenylic acid, ATP and cozymase). It was found that, although the pH of the solution had been carefully adjusted, many cytolyzed cells were extruded in the case of Ambystoma explants, in all the experiments. No visible cytolysis however, occurred in the case of *Rana palustris* explants. The actual results of these experiments will, of course, only be known when the material has been sectioned and studied microscopically, but it is obvious that nucleic acids and nucleotides are toxic for gastrula cells even in relatively small concentrations.

TABLE 1

Type of Implant	No. of Evocations	Total No. of Experiments	% of Evocations	Ribonucleic Acid Content of Implant %
Liver granules	36	52	69	1
Tobacco mosaic virus	26	37	70	1
Yeast granules	10	16	62.5	1.1
Kidney granules	15	28	53.5	0.8
Gastrula granules	13	35	37	0.4
Supernatant fluid from kidney	9	38	24	0.1
Supernatant fluid from gastrulae	6	31	19	0.2
Supernatant fluid from liver	5	47	10.5	0.1
Liver granules plus ribonuclease	3	58	5	Traces
Tobacco mosaic virus plus ribonuclease	0	20	0	Traces

Granules = pellet from ultracentifugation fixed with alcohol and washed with sterile saline.

Supernatant = Liquid from ultracentrifugation precipitated with alcohol.

Since cytolysis is such an important factor in the evocator problem, it would be interesting to know what changes occur in the ribonucleic acid content of the cytolysing pieces of ectoderm. So far, the problem has only been attacked with cytochemical methods, which show that the basophilia of cells which are exposed to an abnormal saline solution first markedly increases. As in normal cells, the basophilic material is digested away by a dilute solution of crystalline ribonuclease. This increase in basophilia is largely due to the fact that, at the onset of cytolysis, the ribonucleic acid-rich cytoplasm separates itself from the yolk; the cells look exactly as they do after centrifugation. It is possible, however, that the increased basophilia may also represent an actual synthesis of ribonucleic acid when cytolysis is just beginning, and the question will remain unsettled until quantitative ribonucleic acid estimations have been done. But, after this initial period of strong basophilia, the cells become more pigmented and simultaneously lose their affinity for basic dyes. At the time of the breakdown of the membrane, only the nucleus still stains with basic dyes. The changes in basophilia during cytolysis are thus identical with those which have been recorded earlier in the case of grafted killed tissues. The strongly basophilic ribonucleoproteins are possibly broken down in both cases until a point is reached where staining ability entirely disappears. It may thus very well be that in cytolyzing ectodermal cells, as in the case of the grafted organs or nucleoproteins, the evocating substance is a breakdown product of ribonucleic acid. The similarity between the two cases is certainly very striking.

C. *Nucleic Acid Distribution in the Normal Embryo*

The localization of nucleic acids in the Amphibian egg during its development has been studied with the Feulgen test for desoxyribonucleic acid and with the ribonuclease method for ribonucleic acid (Brachet, 1940, 1942). The basic dyes used in the latter technique were either toluidine blue, which gives sharper results in earlier stages, or Unna's pyronine-methyl green mixture which is more advantageous when the nuclei become very numerous (in neurulae and later stages), because it brings out a clear-cut difference between the green nuclei and the red cytoplasm. There usually is a slight staining of the yolk with either toluidine blue or pyronine; this basophilia of the yolk is not affected by ribonuclease, and is presumably due to phosphoproteins.

Holtfreter has recently pointed out that fatty acids present in the 'lipochondria' stain with basic dyes and that lipids might thus interfere with the detection of ribonucleic acid in Amphibian eggs. However, it should be emphasized that proper staining of nucleic acids occurs only when fixing fluids which remove the lipids are used. The presence of lipids actually reduces the basophilia due to ribonucleic acid, as shown by Monné. Furthermore, we have found that continuous extraction for 14 hours in a boiling ether-alcohol mixture in a Soxhlet apparatus, completely fails to change the basophilia. Since it has been suggested by S. Cohen that mononucleotides may be attached to proteins and digested away by proteolytic impurities present in ribonuclease, sections of Amphibian eggs were extracted in cold trichloracetic acid. No change in basophilia was observed after this treatment. It is also of interest to note, in this respect, that the proteolytic activity of the crystalline ribonuclease preparations used in these investigations seemed negligible when used in cytochemical tests. As a control, sections were treated with ribonuclease in the usual way and then subjected to Serra's method for the detection of tyrosine, and that described by Thomas for arginine. No visible decrease in the intensity of the reactions occurred, although their affinity for toluidine blue had entirely disappeared. It was also found that heating of the sections in 10% trichloracetic acid at 90° C for 15 minutes, a treatment found by Schneider to remove entirely both types of nucleic acids, led to the total disappearance of basophilia. Since, as will be shown later, good agreement was found to exist between the cytochemical findings and microchemical estimations of pentoses in various parts of the egg, there seems to be no reason to doubt that the basophilic material observed really corresponds to the nucleic acids of the cell.

The young, yolkless oocytes are very rich in ribonucleic acid, as shown by their strong basophilia and by pentose analyses. In later stages, the ribonucleic acid content decreases along an animal-vegetal gradient. If fully grown oocytes are centrifuged at moderate speeds, the yolk is packed at the centrifugal pole, while the ribonucleoproteins accumulate at the centripetal end. It is interesting to note, in view of the theory presented by Dalcq and Pasteels to explain morphogenesis, on the basis of an interaction between a cortical field, unaffected by centrifugation, and a yolk gradient, that these centrifuged oocytes show a cortical layer of ribonucleoprotein which is not moved by centrifugation. Furthermore, the presence of a yolk gradient becomes very obvious when reactions for tyrosine and especially arginine are applied to sections of normal oocytes. Apparently the large vegetal yolk platelets are richer in these amino acids than the small platelets at the animal pole.

The ribonucleoprotein gradient decreasing from the animal to the vegetal pole, remains the same after fertilization and during the segmentation of the egg. At the latter stage, the cortical layer forms a thin membrane around the blastomeres. At gastrulation, the presence of a second gradient, this time decreasing dorsoventrally, becomes obvious. There

is a distinct difference in affinity for basic dyes between presumptive neural system and presumptive epidermis, as well as between the organizer and the ventral mesoblasts.

At late gastrulation stages, the gradient patterns show further complications. In the presumptive neural plate, a distinct gradient, decreasing anteroposteriorly, is visible while in the underlying chordamesoderm an opposite gradient can be found. It would seem that ribonucleic acid disappears during invagination. A distinct accumulation of ribonucleic acid is found at the place where the ectodermal and chordal cells come into close contact.

TABLE 2

	Ribo-nucleic acid	Desoxyri-bonucleic acid	ATP gamma/ 100 gammas N
Dorsal half of gastrula	7.6	7.6	2.5
Ventral half of gastrula	5.9	8.4	1.3
Dorsal half of neurula	10.5	12.7	3.8
Ventral half of neurula	5.7	8.3	1.15

Finally, during neurulation, the axial organs are obviously the site of a strong synthesis of both types of nucleic acids. The nervous system stands out as being very basophilic, in contrast to the ectoderm, while a very distinct gradient can be observed in the chordamesoderm. The amount of ribonucleic acid disappears in a very gradual way from chorda to somites to future pronephros and lateral plates. It is worth mentioning that this cytochemical gradient exactly duplicates the morphogenetic gradient discovered by Yamada.

Chemical estimations of the ribo- and desoxyribonucleic acid content have recently been made on isolated dorsal and ventral halves of early gastrulae and early neurulae. As was to be expected from the cytochemical observations and as shown in Table 2, an appreciable synthesis of both nucleic acids occurs only in the dorsal half. But, even in the gastrula stage, more ribonucleic acid is present in the dorsal half. It is also worth noting that ATP shows the same behavior as the ribonucleic acid (Brachet and Chantrenne, 1942).

It is thus obvious that ribonucleoprotein is distributed in the egg according to a definite gradient, which essentially follows the "morphogenetic potentials" (Dalcq and Pasteels) of the egg. These gradients are very similar to the respiratory and reduction gradients of the various parts in the egg, a finding which is not surprising in view of the presence in the same granules of important respiratory enzymes (cytochrome oxidase and succinic dehydrogenase) and ribonucleoprotein (Stern, 1939; and Chantrenne, 1943). It is further known from Boell's work on Ambystoma that the growth of these two respiratory enzymes is parallel to the increase in the oxygen consumption; it is likely that their synthesis is an index of the rate of the transformation of yolk reserve into 'active protoplasm'. Such a finding is, of course, to be expected in view of the constant association between a high ribonucleic acid content and protein synthesis (Caspersson, Brachet).

D. *Ribonucleic Acid Synthesis and Morphogenesis in Experimentally Modified Conditions*

Various attempts have been made to ascertain whether ribonucleic acid synthesis and morphogenesis remain closely linked to each other when conditions are altered by experimentation. So far, experiments have been done by interfering with nucleic acid synthesis by the addition to eggs of chemical analogues of the purine or pyrimidine bases, by centrifugation of the egg at various stages and by studying parthenogenetic, polyspermic and hybrid embryos.

It is possible to interfere with nucleic acid synthesis in bacteria or yeast by addition of barbituric acid (which corresponds to uracil) (Woods, 1942), of benziminazole, which is a chemical analogue of adenine (Woolley, 1944), or of acriflavine, which combines with nucleic acids and nucleotides to form insoluble complexes (McIlwain, 1941). If Amphibian blastulae or gastrulae are treated with these chemicals in proper concentrations, it is found that the embryos retain a spherical shape while the controls normally elongate. Microscopic and microchemical observations further show that neural induction is blocked to a considerable degree, especially in the posterior part of the embryo, while nucleic acid synthesis is definitely slowed down. It should, however, be added, that we were unable to prove that barbituric acid and benziminazole act in a specific way, on nucleic acid synthesis, and not as ordinary narcotics, because both development and nucleic acid synthesis are resumed when the eggs are returned to water. Thus, no addition of uracil or adenine is required to obtain a reversal of the antagonistic effect, as is the case in bacteria. But such a result was to be expected, since the embryo is obviously able to synthesize purine and pyrimidine bases from its yolk, not requiring these substances in the external medium. Furthermore, in the case of acriflavine, which makes an insoluble complex with the nucleotides, a beneficial action of added adenylic acid was observed several times.

The experiments on centrifugation which were carried out in collaboration with Dr. Pasteels, show that ribonucleoproteins are shifted towards the animal pole if the egg is centrifuged shortly after fertilization. Such a modification of the normal animal-vegetal gradient provides a satisfactory explanation of the inhibition of morphogenesis noted (Pasteels). When the eggs are centrifuged in the

blastula stage, the blastocoele roof collapses and comes into contact with the yolk-rich endodermal cells. In all cells of the blastula, ribonucleoprotein accumulates at the centripetal end, so that the ectoderm is in close contact with an abnormally basophilic layer of cytoplasm. The result of such an experiment is the production of double and sometimes triple embryos, and it may be that the induction of these accessory embryonic axes is due to these localized accumulations of ribonucleoprotein materials. At any rate, a very constant feature of these embryos is that a considerable synthesis of both types of nucleic acid takes place in the secondary neural tubes just as it does in the primary ones. These experiments, as well as others performed on a smaller scale on exogastrulae and explants, indicate clearly that one of the constant characteristics of the normal induction is that the ectoderm, while it is being transformed into a medullary plate, is the site of marked nucleic acid synthesis.

Comparable results were obtained in a cytochemical study of the nucleic acid localization in parthenogenetic and polyspermic embryos of *Rana fusca* (Brachet, 1945). Dispermic eggs are especially useful, since it often happens that exactly one half of the embryo is haploid and the other diploid. In such embryos, it is found that the total amount of nucleic acid in, for example, a haploid somite and the corresponding diploid one is very much the same. Apparently, the individual haploid cells contain one half as much ribo- and desoxyribonucleic acid as the diploid ones. But the whole haploid somite contains twice as many cells as the other. Where, however, the embryo is asymmetrical because the development of one side (usually the haploid one) has lagged behind the other, ribonucleic acid synthesis is always delayed in the hypomorphic side. There is thus another close correlation here between nucleic acid synthesis and morphogenesis.

Finally, a lethal hybrid combination (*Rana esculenta* ♀ × *Rana fusca* ♂), the development of which stops during gastrulation, has been studied. Nucleic acid synthesis also stops at gastrulation, or, more exactly, only the early phase of ribonucleic acid metabolism occurs. The cytoplasmic basophilia decreases, due probably to a conversion into desoxyribonucleic acid. But the synthesis of new ribonucleic acid during gastrulation, which is possibly linked with increased protein synthesis at that time, does not take place. It seems, then, that the presence in the cytoplasm of an abnormal set of chromosomes prevents the ribonucleic acid synthesis. If, however, the organizer from the blocked gastrula is grafted into the blastocoele of a normal diploid Triton embyro, both development and nucleic acid synthesis are resumed in 50% of the cases. The graft differentiates into chorda cells which contain a very basophilic cytoplasm and nuclei, and which induce a secondary neural tube in the host. This resumption of development failed to occur in the other 50% of the cases. The graft retained its typical gastrula cell structure, and no visible synthesis of nucleic acids occurred. Here, again, morphogenesis and nucleic acid synthesis were closely linked.

E. *General Considerations*

The work which has just been summarized strongly suggests that ribonucleic acids or some of its derivatives play an important part in the induction of the neural tube. It would, however, be very exaggerated to state that 'the organizer is a nucleic acid'. The induction is without doubt a complicated process, involving a number of chemical reactions and, probably, physical processes. The gradient system certainly plays an important role in the regional differentiation of the nervous system.

Two possibilities, which will now be briefly discussed, seem to be the most likely to provide an explanation of the behavior of ribonucleic acid at the time of induction. One of them is that ribonucleic acid is broken down during invagination into mononucleotides, which would diffuse into the presumptive neural plate and be used there as a raw material for synthesis of nucleic acids and ATP. Local synthesis of these substances could possibly in turn lead to synthesis of new specific proteins in the neural plate. Such a hypothesis has some advantages. It accounts for the decreasing cephalo-caudal ribonucleic acid gradient in the invaginated chordamesoderm; furthermore, this mechanism is probably the same which is at work when abnormal evocator (killed tissues) or cytolyzing cells are responsible for evocation. In all these cases, ribonucleic acid is broken down in the evocator and there is simultaneously a synthesis of the same substance in the neighboring epidermis. We tried recently to put this hypothesis to a test by placing a cellophane sheet, which would allow the diffusion of nucleotides but not of polymerized nucleic acids, between an organizer and epidermis, but the results of these experiments will only be known when a thorough cytological study of these explants has been made.

The second hypothesis is that the important factor is not ribonucleic acid itself, but a whole 'nucleoproteic granule' complex, containing respiratory and hydrolytic enzymes. Such a granule might conceivably act in differentiation as a plasmagene or virus-like particle. The existence of such granules is known from Claude's work, as well as from our own. It is interesting that in unfertilized frog eggs a large proportion (over 50%) of the ribonucleic acid cannot be sedimented by centrifugation at 100,000 × g for 20 minutes, while in tadpoles, as well as in adult tissues, most of the ribonucleic acid is associated with centrifugable particles (Brachet and Chantrenne, 1943). Recent work by Chan-

trenne (1947) has shown that granules isolated from liver by differential centrifugation vary in composition in such a way that the smallest particles are almost pure ribonucleoprotein while the larger ones contain many enzymes. The possibility is thus opened that these nucleoproteic granules, during embryonic development, become larger and more complex. One might conceive that the unfertilized egg contains mostly small, nonspecific granules, which become, perhaps under genetic control, quantitatively and even qualitatively different in various parts of the egg, as development proceeds. Such a hypothesis would be in good agreement with the results we obtained with the hybrids, where a nuclear control of ribonucleic acid synthesis is suggested by the experimental results. Furthermore, the idea that the basophilic material of the embryo is made of ribonucleoprotein associated with the enzymes is strengthened by the fact that the gradient in basophilia is identical with that of oxygen consumption. Quite recently, it has been found by Dr. E. Krugelis that the same gradients are also demonstrable in the case of alkaline phosphatase, an enzyme which is known to be present in the ribonucleoproteic granules of many tissues.

It is also of interest, in this respect, to remember that it has been shown that in one case of virus infection, at least, (that of silk-worm jaundice) studied by Gratia, Jeener and Brachet, the multiplication of the virus induces a considerable synthesis of ribonucleic acid in the infected cells. The possibility that nucleoprotein granules may move the from the organizer to the ectoderm is not to be rejected wholly, since Holtfreter showed that embryonic cells are able to ingest particles of carbon or carmine. Furthermore, we observed recently that when an organizer stained with neutral red is grafted into the blastocoele of a young gastrula, the ectoderm definitely takes some of the stain, which is, however, only present in the yolk platelets, pigment granules and in the nucleoproteic granules, or, in other words, in microscopically or ultramicroscopically visible particles.

However, preliminary experiments made with the help of Mr. Shaver in order to show the 'virus-like' activity of the nucleoprotein granules, have so far been negative. The particles were isolated by ultracentrifugation of extracts from the archenteron roof of neurulae, and injected in ventral blastomeres of morulae or blastulae. In no case can a secondary medullary plate be made out when the host survives to the proper stage, but it is obvious that these negative experiments are insufficient to disprove the hypothesis entirely.

Although we are still far from a satisfactory understanding of the mechanics of neural induction, it may be hoped that the continuation in the future of a concerted attack, using cytochemical and microchemical methods, will prove highly fruitful.

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DISCUSSION

CHARGAFF: I wonder whether one group of substances present in egg yolk has not been somewhat neglected in the course of these discussions. I refer to the phosphoproteins of the type of vitellin. Although these proteins usually occur as lipoproteins and may in this form be unable to combine with basic dyes, there could conceivably arise a situation in the course of development where they could stain basophilic. Furthermore, the phosphoserine groups abundant in vitellin might play a role in transphosphorylation because of their possible transformation into phospho-hydroxypyruvyl groups. In experiments with radioactive phosphorus which we carried out some time ago we found that vitellin was formed in the egg with much greater speed than any other phosphorus compound.

BRACHET: We know that the frog's egg yolk contains phosphoproteins in addition to small amounts of ribonucleoproteins. This yolk is very slightly basophilic and this basophilia remains unaffected by ribonuclease. It is very likely that the phosphorus of the yolk phosphoproteins is used during development for the synthesis of nucleic acids. It has been found independently by Harris and by myself that the yolk platelets contain a specific phosphatase which attacks only phosphoproteins (phosphoproteinphosphatase). It may be that inhibition of this enzyme, which certainly deserves further study, by inorganic phosphate, may account for the inhibition of morphogenesis which I recently observed in eggs treated with fairly concentrated (M/30 to M/60) phosphate solutions.

VILLEE: I have been raising Drosophila on a

chemically defined medium similar to that described by Schultz, and varying the nucleic acid fraction. The larvae can grow, but only very slowly, on a medium containing no ribonucleic acid, which indicates that they can synthesize this completely. The length of time from hatching to pupation can be greatly shortened by the addition of ribonucleic acid, a mixture of nucleotides obtained from ribonucleic acid by hydrolysis, or by adenylic acid or adenine. When benzimidazole is added to ribonucleic acid in the medium, the larvae hatch but are unable to grow, and die in about two days. There is thus an antagonism between benzimidazole and ribonucleic acid in the growth of Drosophila similar to that described by Dr. Brachet. When benzimidazole is added to the medium in place of ribonucleic acid, however, the larvae grow and pupate in about the same length of time as on ribonucleic acid.

McDonald: Dr. Brachet has mentioned the possible interference of proteolytic enzymes with his histochemical test for ribonucleic acid. Many of the samples of crystalline ribonuclease which have been used for this purpose have undoubtedly had proteolytic activity. We have succeeded in preparing ribonuclease free from all measurable traces of proteolytic enzymes. Using such samples of ribonuclease, Dr. Kaufmann has found it possible to remove all of the pyronin-staining components of both cytoplasm and nucleus. In experiments in which "proteolytic-free" and "proteolytic-contaminated" samples of ribonuclease were compared, no differences in gross cytological results were observed. It would therefore seem that the removal of pyronin-staining material by ribonuclease is not due to proteolytic contaminants.

Doyle: There is a correlation between the time of onset of increase in mass of the developing sea urchin and the time at which the total content of peptidase activity of the sea urchin begins to rise as shown by Holter and Lindahl. This time coincides with the period at which ribonucleic acid synthesis, as evidenced by basophilia, has been observed by Dr. Brachet to begin in the sea urchin.

In the data presented on differences between dorsal and ventral halves of the frog embryo there is a quantitative difference in content with respect to both nucleic acids. Since the individually larger cells of the ventral half all contain "inert" yolk one wonders whether the nucleic acid content of the clear cytoplasm of both halves may not be essentially identical.

Brachet: We have no data as yet on the relative amounts of RNA in the "clear cytoplasm" in various parts in the embryo; but it must be pointed out that this synthesis of clear cytoplasm at the expense of the yolk, at different rates in the different regions of the embryo, might well be an important morphogenetic factor. As a matter of fact, such a transformation would agree very well with the Dalcq and Pasteel theory of development, where interaction between a cortical "field" (which seems to contain ribonucleic acid) and the yolk gradient leads to the formation of unequal amounts of a morphogenetic substance; we believe the latter to be of ribonucleoproteic nature.

Concerning the linear shape of DNA synthesis in the sea urchin egg, it should be made clear that this linearity represents only an approximation; unfortunately, accurate measurements are impossible in the early stages of segmentation, because the DNA content is still very low as compared to protein and ribonucleoprotein. It should be borne in mind that at later stages the situation becomes complicated by the facts that the volume of the chromosome decreases and that the mitoses are no longer synchronous.

ON THE NUCLEOPROTEINS AND NUCLEIC ACIDS OF MICROORGANISMS

ERWIN CHARGAFF

INTRODUCTION

The difficulties confronting the cytochemist in his attempt to study the composition of indigenous cellular constituents are multiplied in the case of microorganisms, owing to limitations of size and availability of material. It is, therefore, rather remarkable that interest in microbial nucleoproteins and nucleic acids extends to the very beginning of our knowledge of this subject. This early attention is undoubtedly due to the acuteness of two men, Miescher and Hoppe-Seyler, who in a flash of insight rarely equaled in the history of the biological sciences recognized the importance of this group of substances almost immediately after their discovery in animal and plant cells. Following the isolation by Miescher of nucleic acid from pus cells, Hoppe-Seyler repeated these experiments and at the same time reported the discovery of a similar compound in yeast (Hoppe-Seyler, 1871).

GENERAL REMARKS

Before proceeding to a brief discussion of some of the recent work on bacterial nucleoproteins and nucleic acids I should like to make a few remarks of a more general nature. With respect to the types of nucleic acids encountered in microorganisms, I do not think that any organism has been shown to contain only one of the two main groups. It is most likely that by more refined methods both pentose nucleic acid and desoxypentose nucleic acid will be found in all cells, although their relative proportions may vary considerably depending upon the bacterial species and the developmental stage of the cells. It is, however, possible that the sugar constituents will not be the same in some instances and designations such as desoxyribose or ribose nucleic acid should be avoided, unless the sugar has been identified. With the exception of yeast ribose nucleic acid, this has not yet been possible for any microorganism. This nucleic acid, the prototype of all known pentose nucleic acids, will be fully discussed by other speakers at this symposium and will, therefore, not be further considered here.

It cannot yet be decided whether nucleic acids of the same sugar type, but derived from different species, differ structurally. One set of findings which will be mentioned later, namely the activity of desoxypentose nucleic acids of pneumococci and *E. coli* as transforming agents, points strongly in this direction. But the chemical basis of specificity remains obscure. I shall return to this point at the conclusion of this paper.

In the few cases where the nature of the nitrogenous constituents of bacterial nucleic acids was determined, the purine and pyrimidine composition appeared to be similar to that of nucleic acids of animal and plant cells. There is one possible exception: 5-methyl cytosine has been claimed as a constituent of tuberculinic acid, the depolymerized desoxypentose nucleic acid isolated from *M. tuberculosis* (Johnson and Coghill, 1925). It is unfortunate that no independent confirmation of this potentially very interesting finding has come forth so far.

Nothing is known concerning the position of phosphoric acid on the sugar moiety of bacterial nucleic acids. The assumption of a 3-phospho sugar is almost entirely based on analogy. But a report of LePage and Umbreit (1943) is of the greatest interest in this connection. These authors studied the structure of phosphoribose samples obtained from adenosine triphosphate isolated from a number of microorganisms. The samples obtained from *E. coli, B. subtilis, Staph. aureus,* and yeast all yielded 5-phosphoribose as is found in the ATP of animal tissues. *Thiobacillus thioöxidans,* on the other hand, appeared to contain 3-phosphoribose.

The nature of the protein components of bacterial nucleoproteins is completely unknown. It cannot even be decided whether substances of the histone or the protamine groups are among them, in spite of statements in the literature which will usually be found to have been based on dubious analogies with animal nucleoproteins. Since our conception of the latter group of compounds, especially of the nucleohistones, appears due for a revision, a discussion of the microbial proteins found in combination with nucleic acids is unprofitable.

RECENT WORK ON NUCLEIC ACIDS AND NUCLEOPROTEINS

Bacterial nucleus

Do bacteria contain a nucleus? This question has agitated many workers; but at the present moment it appears no more meaningful than the everlasting controversies about the authorship of Shakespeare's plays which were finally resolved by the recognition that another fellow by the same name must have been the author. The important property of the nucleus of animal and plant cells is not so much its appearance or shape as the orderly changes, connected with definite biological tasks, which it can be seen to undergo. Observations on karyo-

kinetic phenomena in the bacterial cell certainly have not yet found general acceptance as will be shown by a comparison of the noncommittal character of the chapter on the cytology of bacteria in the book of Dubos (1945) with the very impressive evidence provided, in the same book, by Robinow (Addendum in Dubos, 1945). (Compare also Peshkov, 1944-45).

It is, however, fairly certain that the bacterial cell possesses mechanisms that resemble in their function, regardless of their morphological appearance, the nuclear apparatus of higher cells. The epochal experiments by Avery and his associates on the transformation of pneumococcal types, reviewed recently by McCarty (1946), have emphasized the very important role played by some bacterial nucleic acids in the determination of inheritable synthesizing abilities.

It will hardly pay to catalogue all the work done in the past on microbial nucleic acids since, with a few exceptions, very little information about their composition and specific differences has come to light. Moreover, most of the older work was marred by inadequate and over-robust isolation methods (strong alkali, heat, etc.) and must have led to sorry fragments of these bacterial components. The importance of the intactness of the architecture of the nucleic acids may be gathered from the ease with which the transforming factor of pneumococci is inactivated by enzymatic depolymerization (McCarty and Avery, 1946a) and even by drying in the frozen state (Avery, MacLeod, and McCarty, 1944). I shall, therefore, limit myself here to a very brief survey of some of the recent work published within the last twelve years. (The older literature has been reviewed briefly by Porter, 1946, and by Boivin, 1942.)

Nucleoproteins

We still lack a systematic approach to the isolation of the nucleoproteins and intact nucleic acids of microorganisms. Each bacterial species represents a new problem for which a specific solution must be found. Certain general precautions will, of course, have to be observed, *e.g.*, for the isolation of native desoxypentose nucleoproteins the avoidance of agents, such as strong salt solutions, which break or alter the non-electrostatic bonds between nucleic acid and protein; for the isolation of desoxypentose nucleic acids the inhibition of the depolymerizing enzymes.

For the reasons mentioned above, no reliable estimates of the nucleoprotein content of bacteria can be given. The decision as to what constitutes a genuine nucleoprotein rests on chemical and physical criteria which cannot be applied to the intact bacterial cell. It would almost make as much sense to consider the entire cell as one gigantic particle of a lipo-muco-nucleoprotein; but that would not get us very far. The careful work of Menzel and Heidelberger (1938 a, b) on the proteins isolated from tubercle bacilli has provided indications of a high nucleoprotein content in these organisms. Belozersky (1939, 1940) estimated that in a large number of bacterial species the nucleoproteins constituted 50% to 80% of the cell mass. Similarly, the nucleoprotein content of hemolytic streptococci has been estimated to lie around 80% (Sevag, Smolens, and Lackman, 1940).

Nucleic acids

The estimates of the amounts of nucleic acids present in bacteria are perhaps slightly more reliable, though the significance of individual values remains doubtful since the abundance of pentose nucleic acids has been reported to increase in proportion with the rate of growth (Boivin and Vendrely, 1943). Streptococci were found to contain 15% to 24% of nucleic acids; 10% to 30% of the nucleic acids belonged to the desoxypentose type (Sevag, Smolens, and Lackman, 1940). *Proteus vulgaris* and *Myxobacterium sorangium* contain about 13% of nucleic acids of both the pentose and desoxypentose varieties (Belozersky, 1939). The study of staphylococci, typhoid bacilli, and *E. coli* revealed the presence of 3% to 4% of desoxypentose nucleic acid and of 5% to 10% of pentose nucleic acid (Vendrely and Lehoult, 1946). Boivin and Vendrely (1946) found a smooth strain of *E. coli* to contain 13.1% of total nucleic acids, 4.4% of the desoxypentose type.

A few other instances where the presence of both nucleic acid types was noted may also be mentioned: in *Bact. typhi-murium* (Akasi, 1938); in the nucleoproteins of hemolytic streptococci (Zittle, 1939) and of gonococci (Stokinger, Ackerman, and Carpenter, 1944); in the nucleoproteins of several species of Brucella (Stahl, Pennell, and Huddleson, 1939); and in a number of acid-fast bacilli (Petrik, 1946).

Information on the nature of the constituents of microbial nucleic acids has been extremely scanty during the past few years. As was mentioned before, with the exception of yeast ribose nucleic acid no sugar constituent has been identified unequivocally. The nitrogenous components likewise have been examined infrequently, apart from often misleading group reactions. This is the more regrettable since experience with the chemistry of other fractions of the bacterial cell, especially of pathogenic organisms, would almost lead us to expect the unexpected. Ferramola (1935) reported the identification of adenine and guanine in the nucleoprotein fraction of *B. anthracis*. Mitra (1936) isolated cytosine and uracil from the nucleic acid of *Vibrio cholerae* and reported thymine absent. The presence of guanine, adenine, cytosine, uracil, and thymine in the nucleic acid fraction of *Bact. typhi-murium* was reported by Akasi (1938). Guanine, adenine, and cytosine were found in Brucella (Stahl, Pennell, and Huddleson, 1939).

The isolation of the nucleic acids themselves has

been accomplished rather rarely. A pentose nucleic acid was prepared from *Penicillium glaucum* (Akasi, 1939). The isolation from *Spirillum volutans* of a peculiar pentose nucleic acid containing sulfuric acid in ester linkage has been reported by Belozersky (1941, 1945). The very interesting findings with respect to the role of pentose nucleic acids in maintaining the Gram-staining properties of microorganisms can be mentioned here only very briefly. The autolysis of pneumococci under certain conditions has been found to be accompanied by the release into solution of a mixture of pentose nucleic acid and nucleoprotein and by the loss of the Gram-positive character of the cells (Thompson and Dubos, 1938). Later it was found that a magnesium pentose nucleoprotein was the actual carrier of the Gram stain and that, under certain conditions, the recombination of Mg ribonucleate with the Gram-negative "cytoskeleton" to give a Gram-positive complex could be effected (Henry and Stacey, 1943, 1946; Henry, Stacey, and Teece, 1945; compare also, Bartholomew and Umbreit, 1944).

In a few cases highly polymerized desoxypentose nucleic acids, similar to the thymus desoxyribose nucleic acid of very large particle size which has been made available thanks to the brilliant work of Hammarsten (1924), have been prepared from microorganisms. The transforming substances isolated from Pneumococcus Type III (Avery, MacLeod, and McCarty, 1944) and later from Types II and VI (McCarty and Avery, 1946b) have all been shown to represent specific forms of highly polymerized desoxypentose nucleic acid. Comparable results were later obtained by Boivin and Vendrely (1946) with a desoxypentose nucleic acid from *E. coli*.

The isolation of highly polymerized desoxypentose nucleic acid from yeast, giving very viscous solutions, was reported recently (Chargaff and Zamenhof, 1947). This substance had an electrophoretic mobility of -15.7×10^{-5} in phosphate buffer of pH 7.4. It was highly asymmetric (axial ratio of almost 400), its diffusion constant D_{20} was 1.09 $\times$ 10^{-7}, its specific viscosity (0.115% solution in water at 30.3°) was 5.9. The ultraviolet absorption spectrum of this nucleic acid is reproduced in Fig. 1. (In this figure, as well as in the following ones, the values plotted as the ordinates are given as the molecular extinction coefficient with respect to phosphorus: $\varepsilon(P) = 30.98$ E/cl, where E is the extinction, c the phosphorus concentration of the solution in grams per liter, and l the thickness of the absorbing layer in cm.) We are now studying the chemical composition of this nucleic acid.

Nucleoproteins of tubercle bacilli

At the conclusion of this section I should like to mention some as yet unpublished work carried out in our laboratory on the nucleoproteins of tubercle bacilli (Chargaff and Saidel, 1947). We were for a number of reasons interested in the chemical nature of those water-soluble components of the tubercle bacillus that may be extracted from the bacterial cells by mild means, avoiding drastic operations such as the use of acid, alkali or alcohol. In all these experiments the avian strain of tubercle bacilli was employed.

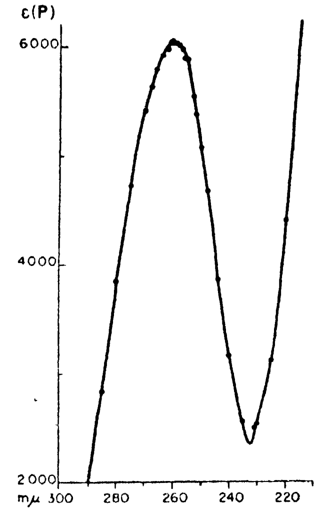

FIG. 1. Ultraviolet absorption of yeast desoxypentose nucleic acid in distilled water.

Tubercle bacilli, like most other microorganisms, are notorious for the difficulty with which they yield their proteins and carbohydrates to extraction. It is doubtful whether this is only due to a particular toughness of the bacterial membrane. One almost gets the impression that the bacterial cell represents one huge complex in which the several constituents are interconnected in such a manner as to make their detachment without far-reaching chemical changes practically impossible. The apparent absence from the bacterial cell of components of low and intermediate molecular weights may, of course, be attributed to the unicellular nature of the material. In contrast to animal tissues, there can be no intercellular substances; degradation and excretion products will be found primarily in the culture medium. In the case of the tubercle bacillus this corresponds to the tuberculin which, it is of interest to note, has been shown to contain considerable amounts of desoxypentose nucleic acid (Seibert, 1940; Seibert and Watson, 1941).

A considerable number of methods for the disintegration and extraction of tubercle bacilli were tried, in general without conspicuous success. I might mention ultrasonic treatment, grinding between rotating glass cones, incubation with crystalline trypsin, extraction with diethylene glycol. The best results were obtained when the bacteria, which were grown on the synthetic Sauton medium, were ground with very fine Pyrex glass powder and extracted with borate buffer of about pH 8.4. The

extracts thus obtained are compared in Table 1 with those prepared by treatment of the crushed organisms with a 10% sodium chloride solution. The slowest fraction corresponds in both cases to the bacterial glycogen of the very high particle weight of 12 million described previously (Chargaff and Moore, 1944). The component moving with a

TABLE 1. ELECTROPHORESIS OF AVIAN TUBERCLE BACILLUS EXTRACTS
(Barbiturate Buffer, pH 7.7, descending boundaries)

Fraction	Electrophoretic mobilities			
	Borate buffer		10% NaCl	
I	−1.7	55%	− 1.4	70%
II	−7.2	45%	− 7.0	20%
III			−10.1	10%

mobility of about −7 is in both cases the crude nucleoprotein fraction. Sodium chloride extracts gave, in general, much more complex and difficultly separable mixtures. Moreover, the borate extract contained 13.6% of the total bacterial nitrogen, the NaCl extract only 7%.

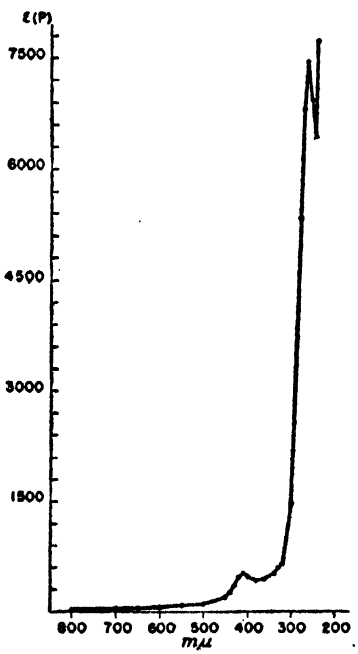

FIG. 2. Absorption spectrum of crude nucleoprotein of avian tubercle bacilli in 0.03 M borate buffer of pH 7.9.

Following the removal of the glycogen by centrifugation at 31,000 g, the borate extracts usually showed only one moving boundary on electrophoresis. The crude nucleoprotein fractions recovered from the dialyzed solution by drying *in vacuo* in the frozen state were slightly yellowish, due to the presence of small amounts of a yellow pigment with

TABLE 2. NUCLEOPROTEIN FRACTIONS OF AVIAN TUBERCLE BACILLI

Fraction No.	Yield	N	P	Nucleic acids (as per cent of total nucleic acid P)	
				Pentose NA	Desoxypentose NA
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
2	2.4	9.1	0.93	41	59
3	2.7	10.0	0.72	35	65
44	0.4	12.1	3.2	13	87

a bluish fluorescence. The complete absorption spectrum of a crude nucleoprotein preparation is illustrated in Fig. 2. These proteins gave strong

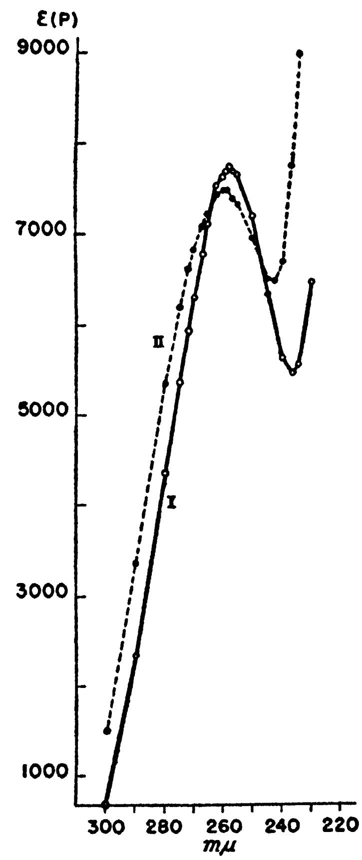

FIG. 3. Ultraviolet absorption spectra of purified (Curve I) and crude (Curve II) nucleoproteins of avian tubercle bacilli in 0.03 M borate buffer of pH 7.9.

color reactions for the presence of desoxypentose nucleic acid.

The crude preparations could be fractionated further. The separation procedure made use of the fact that the bacterial nucleoprotein was found insoluble at pH 4.3, but could not be precipitated by half-saturation with ammonium sulfate. The analytical composition of crude and purified nucleoproteins and the nucleic acid distribution in these fractions are summarized in Table 2. (Fractions 2 and 3 represent crude nucleoprotein preparations; Fraction 44 is a purified specimen.) It would seem that in the course of fractionation the proportion shifted in favor of desoxypentose nucleic acid and that the nucleoprotein precipitated at pH 4.3 consisted almost exclusively of a desoxypentose nucleoprotein. The ultraviolet spectra of a crude and a purified preparation, compared in Fig. 3, were quite similar.

The prolonged dialysis of the nucleoprotein against 1 M NaCl did not produce any cleavage, nor was it possible to precipitate any basic protein by means of flavianic acid. No indications of the presence of a basic protein were obtained. The nucleic acid portion could be split off by treatment with saturated NaCl or with sodium desoxycholate. Its chemical composition and properties are being investigated at present.

A Few Concluding Observations

If, as we may take for granted on the basis of the very convincing work of Avery and his associates, certain bacterial nucleic acids of the desoxypentose type are endowed with a specific biological activity, a quest for the chemical or physical causes of these specificities appears appropriate, though it may remain completely speculative for the time being.

The simplest explanation, but one particularly difficult to test, would consist in the assumption that these nucleic acids contained some hitherto unsuspected chemical constituents that conferred specificity on them. Since, however, there is no reason to consider the nucleic acids isolated from a bacterial species as functionally homogeneous, the desoxypentose nucleic acid molecules active as transforming agents or as biological determinants in any other way may represent a small fraction only of the entire preparation. Even if sufficiently sensitive methods for the isolation of novel nucleic acid components were available, it would be extremely difficult to assign to any of them a specific biological task. In our laboratory, Dr. E. Vischer in collaboration with this writer has embarked on the development of micro separation methods which permit the separation and identification of nucleic acid constituents (purines, pyrimidines, etc.) in individual amounts of as little as 5-10 γ; the separation of the purines or pyrimidines was possible with hydrolysates containing about 200 γ of nucleic acid (Vischer and Chargaff, 1947; and unpublished work). Methods of this type may be expected to contribute to the solution of the problem mentioned here.

Differences in the proportions or in the sequence of the several nucleotides forming the nucleic acid chain also could be responsible for specific effects. The first of these possibilities will be susceptible of proof, once the micromethods alluded to above have been made truly quantitative. The exploration of the second assumption will have to await more knowledge on the mechanisms of hydrolysis of the nucleic acid macromolecules.

Whether the physical state of the nucleic acid preparations (particle weight, degree of asymmetry, etc.) is in any way connected with their biological activity is unknown.

Perhaps the most plausible hypothesis would appear to be one which postulates that biological determination is mediated through a geometrically unique combination between a specific nucleic acid and a specific protein. The forces prevailing in native conjugated proteins are as yet completely obscure. But, as this writer has pointed out some time ago in a discussion of lipoproteins (Chargaff, 1944), the distinction between electrostatic and coordination compounds is probably not sharp in the field of conjugated proteins, since the coordination centers may often be represented by electrically charged groupings in the protein and the prosthetic compound. It is, for instance, conceivable that a particular sequence of the nucleotides in the nucleic acid could make for the accumulation of amino groups (from guanine, adenine, or cytosine) at certain spots in the long chain, thus creating centers that could serve to anchor the nucleic acid in space in a specific manner, once it is combined with a protein. The specific nature of a nucleoprotein would then be vouchsafed not only by the structure of the protein but also by that of the nucleic acid.

But even a better understanding of the geometry of nucleoproteins, from which we are very far indeed, would contribute only little to our insight into the mysterious forces governing the specific synthetic abilities of the cell, since Nature seems to create molds which not only shape the clay but at the same time are shaped by it.

As to the proteins with which the desoxypentose nucleic acids are combined in the bacterial cell, nothing is known about them. There is, in fact, no certainty at all that the isolated nucleoproteins represent true cell constituents and that no switch in the protein partners has taken place in the course of the isolation. If, as appears possible, the growth and the cleavage of nucleic acid chains and of nucleoproteins are important biological phenomena, one could conjecture that in some of the nucleoproteins the protein moiety could be represented by the desoxypentose nucleic acid depolymerizing enzymes themselves and that this biological sequence as so many others (muscle contraction, blood

coagulation) is controlled by a delicate system of checks and balances.

One may object that the considerations submitted above would reduce the genes or what corresponds to them in bacteria to no more than a kink in the nucleoprotein that could hardly be expected to be maintained through innumerable generations. In answer to that I should like to refer to a very elementary experiment in geometry. One of the simplest surfaces studied in topology is the so-called Moebius strip (Courant and Robbins, 1941). It consists of a long paper strip whose two ends are pasted together after one has received a definite number of twists. If, for instance, one end is twisted once completely round (*i.e.* through four right angles) before being joined to the other end and the strip then is cut along the center line, two interlaced rings are obtained each of which has inherited the particular twist. It can again be divided into two interlaced rings, and so on. An inquisitive child, by varying the arrangement, can make many fascinating discoveries about the inheritance of geometrical peculiarities; and when it grows up and remembers them, they may help to take some of the terror from the seemingly automatic nature of the life processes.

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DISCUSSION

BOIVIN: With Dr. Vendrely, I have studied many species of bacteria, including *Mycobacterium tuberculosis:* all contain both desoxyribonucleic acid (from 1 or 2% to 5% dry weight) and ribonucleic acid (from 5 to 10, 15 or sometimes 20%). In chemical analysis we have used, with modifications, the very good method given by Schneider for animal tissues (the use of hot trichloracetic acid). The morphology of bacteria (coccus or bacillus), their behavior with gram stain (gram positive or negative), the virulence of strains and their antigenic constitution (smooth or rough) do not systematically influence the values of desoxyribonucleic and ribonucleic acids. But these values vary greatly with the physiological state of the bacteria: in absolute values, young bacteria are very much richer in ribonucleic acid and slightly richer in desoxyribonucleic acid than the corresponding resting bacteria. By the action of enzymes it is seen that ribonuclease and desoxyribonuclease on fixed bacteria (fixed on slides by formol, etc.) shows different behavior in different species; thus, ribonuclease removes the ribonucleic acid quickly from *Escherichia coli, Ncisseria gonorrhoeae, Corynebacterium diphtheriae* and *Bacillus anthracis,* and slowly from *Staphylococcus aureus.* The explanation probably lies in the uneven firmness of linkage between nucleic acids and proteins, native or denatured. According to Stacey and his co-workers, the gram positive stain is determined by superficial ribonucleoprotein substances. One notes, then, that by action of ribonuclease (on fixed bacteria) the "total" ribonucleic acid of *C. diphtheriae* and of *B. anthracis* (gram positive) is as quickly removed as that of *E. coli* and of *N. gonorrhoeae* (gram negative), while that of *S. aureus* (gram positive) is very slowly removed.

THE SYNTHESIS OF BACTERIAL VIRUSES IN INFECTED CELLS

SEYMOUR S. COHEN[1]

Introduction

All the viruses which have been isolated and studied have been found to contain nucleic acid. The solution of the problems of the isolation, structural relations, specificity and origin of nucleic acids are therefore of great importance in building the body of information necessary to explain the multiplication of a virus. Hence it is not surprising that the entrance of chemists into virology has led to advances in the study of nucleic acid. A few of these special contributions of virology to this field may be noted. The only relatively simple well-characterized proteins containing ribose nucleic acid (RNA) are the plant viruses (Bawden, 1943). The linkages of nucleic acid to protein in the plant viruses are not salt-like, in contrast to linkages in materials such as the nucleoprotamines (*ibid*). The ribose nucleic acid of tobacco mosaic virus was the first representative of the ribose-containing acids shown to have a thread-like structure, whose particle weight was far greater than that of a tetranucleotide (Loring, 1939; Cohen and Stanley, 1942).

The chemical virologist concentrated for more than a decade on the nature of the virus particle. The characterization of nucleic acid, rather than a study of its metabolism was the natural concomitant of such an approach. Accordingly there are practically no data on the metabolism of virus-infected cells in general or of nucleic acid in these cells. Nevertheless, the character of the data amassed on the nature of the virus particle has led inevitably to the consideration of the metabolism of virus-infected cells and of nucleic acid in these cells.

These data were of two types: First, it had not been possible to demonstrate conclusively any enzyme system as a component part of any virus. On the contrary, numerous enzymes were shown to be missing from many kinds of virus particles (Stanley, Knight and De Merre, 1945). Coenzymes essential for the synthesis of virus are either totally missing from the viruses studied or are severely limited in their utilizability by the absence of other interacting groups (Stanley, Knight and De Merre, 1945; Cohen, 1946). These data, although of a negative and partial character, strongly suggest the basis of the parasitism of a virus on its host; *i.e.*, a host is prevailed upon to supply the metabolic enzymatic equipment and energy supply for the multiplication of the virus which invades it. This hypothesis is readily deducible from the primary fact of virology, the multiplication of a virus exclusively within "living" cells. It can only be tested by studying the mechanisms of the multiplication of virus within infected host cells.

Secondly, most, if not all, viruses studied to date contain a single type of nucleic acid (Cohen, 1946). This is in contrast to all cells capable of independent existence which appear to contain two types of nucleic acid. The amounts of this nucleic acid vary widely from 1% to 40% among the viruses and, in many instances, the nucleic acid constitutes the sole phosphorus-containing constituent of the virus (Table 1). It follows, therefore, that other phosphorus-containing constituents are absent from some of these viruses. These additional deficiencies in structures and metabolites underline the enzymatic deficiencies described previously and broaden the gap between cells capable of independent existence and viruses.

Table 1. The P and Nucleic Acid Contents of the Viruses

Virus	Type of Nucleic Acid Found	% Total P in Nucleic Acid
Tobacco mosaic and strains	RNA	Probably 100
Tomato bushy stunt	RNA	
Tobacco necrosis	RNA	
Other plant viruses	RNA	
E. coli bacteriophages (T2, T4)	DNA	99–100
Rabbit papilloma	DNA	>90
Vaccinia	DNA	>95
Epidemic rickettsiae	DNA	No ribose found
Equine encephalomyelitis	RNA	Contain antigens of host
Influenza	DNA and RNA	

However, what deserves special notice here is the splendid tool which a virus supplies for the study of nucleic acid metabolism associated with a specific biological activity. One may select a virus with a readily measurable activity and a large amount of nucleic acid of a single type, containing all of the phosphorus of the virus. It is then possible to follow this special activity and the transformations of nucleic acid and phosphorus leading to the considerable multiplication of activity, nucleic acid and phosphorus. I know of no other system at the pres-

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ent time which is potentially as favorable.

It was stated that most, if not all, viruses studied to date appear to contain a single type of nucleic acid. Two exceptions are reported in the literature and include the T2 bacteriophage and strains of influenza virus. We shall be concerned in detail with the T2 bacteriophage which has been reported by Taylor *et al.* (Taylor, 1946; Hook, Beard, Taylor, Sharp and Beard, 1946) to contain over 40% of desoxyribose nucleic acid (DNA) and smaller variable amounts of RNA depending on the medium of origin. There appear to be serious inadequacies in the identification of RNA by those workers. Regardless of the medium in which this virus originates, we have found at least 99% of the total phosphorus of the virus preparation to be accounted for by the DNA content of the preparation. It will also be shown that the DNA of the virus is a product synthesized during virus multiplication whereas RNA appears practically inert during this process, being neither degraded nor synthesized. It follows from these considerations that it is extremely unlikely that RNA is a constituent of T2 bacteriophage, despite the fact that it is most difficult to exclude absolutely the presence of RNA in material rich in DNA.

Since the presence of only a single type of nucleic acid in a virus is important it might be well to develop the question of the criteria of the identification of nucleic acid. The T2 virus was prepared from lysed bacterial cultures in synthetic media by Taylor and co-workers and analyzed by the method of Schmidt and Thannhauser (1945). These investigators reported the presence of 2% of the total P in a fraction which would ordinarily contain RNA degradation products. Since this amount was so small, and this material of bacterial origin might be expected to present such different conditions for analysis from the mammalian tissues for which the method was devised, it would appear that special precautions were warranted in establishing the presence of RNA in this material. These would include demonstrations of 1) the physical homogeneity of the virus preparations, 2) the absence of antigens of the host, *E. coli,* in the virus preparations since the host is rich in RNA, and 3) a quantitative correlation of purine, pentose, and P in the appropriate fraction and the demonstration that the pentose phosphate is ribose-3 phosphate. In the absence of the last two groups of essential data, the presence of RNA as a component part of T2 cannot be considered proved on the basis of data of the type presented by these workers. Schmidt, Hecht, and Thannhauser (1946) have recently come to this conclusion concerning the inadequacy of a P value in the discovery of the metaphosphate of yeast in the fraction normally containing only RNA nucleotides.

I shall discuss the problem of the nucleic acids in the influenza viruses at a later point in this paper since an approach to its clarification is presented in the results of the study of bacteriophage synthesis.

Advantages of Bacteriophage Systems

We have chosen to study the metabolism of *Escherichia coli B* infected with T2 or T4 bacteriophage to test the hypothesis that a virus utilizes the enzymatic equipment of the host for the synthesis of virus. The body of knowledge which exists on the biological properties of the bacterial virus systems reveals no fact which indicates that the behavior of these systems is radically different from the behavior of viruses infecting plant, animal and bacterial systems (Delbrück, 1946). Many facts suggest a close similarity in the parasitic process in the plant or animal cells. The most striking dissimilarity seems to be that many bacterial viruses have tails. A very active group of workers has accumulated a wealth of data on biological and physical properties of the set of 7 T viruses infecting *E. coli B.* Their cooperative attack has been of great value in the development of the problem.

The viruses studied, the T2 or T4 bacteriophages, appear to possess the general chemical attributes of the viruses. Very similar phages lack enzymatic equipment (Schuler, 1935) and T2 and T4 specifically lack many structures, such as phospholipid and RNA, revealed by the close correlation of ca 40% of DNA and the phosphorus content (Cohen and Anderson, 1946). They may be readily prepared in large amount as follows: *E. coli B* are grown in synthetic medium or nutrient broth to a suitable concentration, infected with a stock of the appropriate strain of virus. Virus multiplies within the bacteria and the host cells are lysed, liberating virus. The lysate is centrifuged at a low speed to remove bacterial debris and subsequently at high speed to sediment virus. Cycles of low speed and high speed centrifugation are repeated once or twice, the resulting products consisting of the characteristic tad-pole shaped viruses, T2 or T4, whose morphologies have been described by Luria and Anderson (1942; see Delbrück, 1946) and Hook *et al.* (1946).

Given a purified concentrate of virus of readily determinable activity, it is then possible to add virus to a given culture of host cells of readily determinable concentration in a medium of accurately known composition at a definite temperature. Thus, every host cell may be infected on the average with a definite number of virus particles. The metabolic changes during virus multiplication under definite environmental conditions may then be followed.

The r^+ Factor

The T2 and T4 viruses are closely related with respect to chemical composition and appearance. They differ in their serological properties, host range, and adsorption to *E. coli B*. It has been

shown, for instance, that many strains of T4 will not adsorb to the host in a synthetic medium devoid of an adsorption cofactor such as tryptophane (Anderson, 1945). T2 does not appear to require a cofactor for adsorption.

Our viruses were chosen because of their unusually high DNA contents which greatly assist the work. Certain genetic complications have appeared, however, which must be described. They have been studied by Hershey (1946) and by A. H. Doerrmann (personal communication). These viruses contain a hereditary factor, termed r^+, which affects lysis in the following manner: an r^+ virus is normally liberated from a cell grown and infected in our synthetic medium (F) in ca 30 minutes. In a dilute suspension of infected cells, essentially maximal liberation occurs at that time. In a concentrated suspension of infected cells, the first small percentage of infected cells that lyse liberate r^+ virus particles which are rapidly readsorbed to the other non-lysed cells. The adsorption of r^+ virus to a cell which already has r^+ virus within it inhibits the lysis of the cell for 70 to 90 minutes more, permitting continuing virus multiplication within the lysis-inhibited cell. Although this complicates the interpretation of results, the phenomenon is of great assistance technically because larger amounts of new substance can accumulate in the extended period of multiplication within the host. The r^+ characteristic may be lost by spontaneous mutation to produce an *r* virus which is not lysis-inhibitory.

Some Additional Characteristics of the T2r^+ and T4r^+ Viruses

These viruses have been prepared from *E. coli* grown on synthetic media (F) and on nutrient broth (N). In Table 2 may be seen some properties of concentrates of these viruses prepared from nutrient broth. T2r^+ from nutrient broth had been claimed by Taylor (1946) to have almost 13% of the total P as RNA-P. It is apparent from the figures in the table that the DNA of T2r^+ or T4r^+ accounts for at least 99% of the total P, considering polymeric DNA to have a P content of 10.0%. It may also be noted that the activity of T4 virus per mg. DNA is considerably higher than that of T2. This is in general characteristic of these viruses; concentrates of T2r^+-N appear to contain a greater number of inactive particles having the general T2 composition than is true of T4. We have found preparations of T2 from the F medium to have atomic N/P ratios of 7.2 to 7.6. T4 from F has had a ratio of 7.9 to 8.0.

Concentrates of these viruses prepared from F media have markedly different properties than the

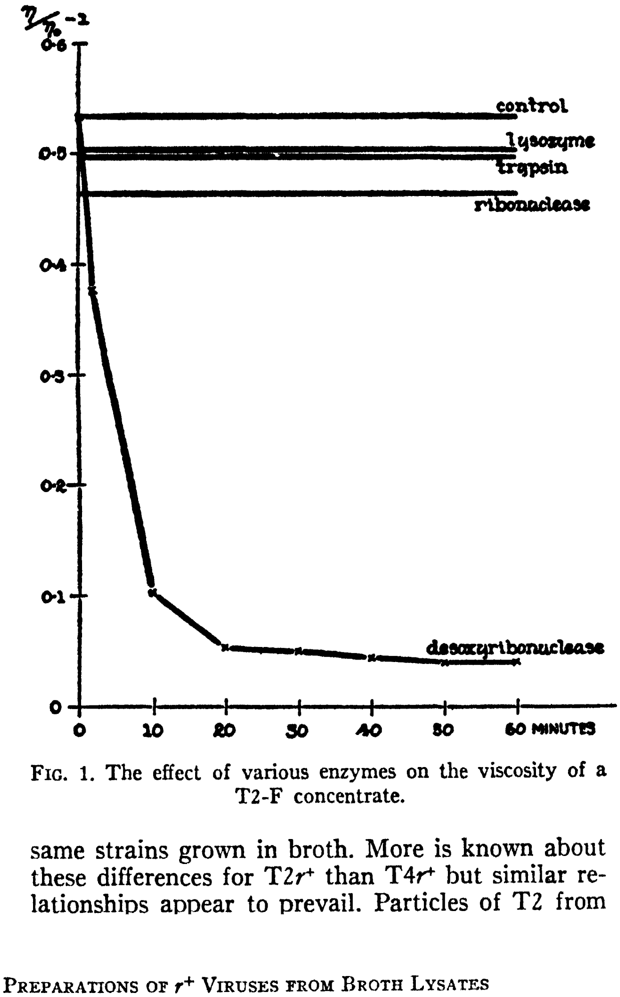

FIG. 1. The effect of various enzymes on the viscosity of a T2-F concentrate.

same strains grown in broth. More is known about these differences for T2r^+ than T4r^+ but similar relationships appear to prevail. Particles of T2 from F are less stable and slightly larger than T2 from broth, according to Hook *et al* (1946). We have found preparations of T2 from F (T2-F) to be less stable and more viscous, than T2 from nutrient broth (T2-N). The viscosity of T2-F appears to be due to a coating of sedimentable DNA, perhaps in the form of nucleoprotein, which facilitates particle interaction, and permits an acid-base interaction with the tribasic antibiotic, streptomycin, to form lattice structures and precipitates (Cohen, 1947). This coating of DNA is not present in T2-N and T4-N and they are accordingly not precipitated by

TABLE 2. COMPOSITION AND ACTIVITY OF PURIFIED PREPARATIONS OF r^+ VIRUSES FROM BROTH LYSATES

Virus	Titer/cc.	mg. N/cc.	mg. P/cc.	mg. DNA/cc.	N/P	DNA−P/P
T_2r^+	4.8×10^{11}	0.105	0.0310	0.306	7.30	0.99
T_4r^+	1.25×10^{12}	0.105	0.0289	0.286	8.07	0.99

streptomycin. It has been found that treatment of T2-F with desoxyribonuclease (Fig. 1) specifically reduces the viscosity of the concentrate without loss of virus activity and converts ca 30% of the total DNA of the preparation into non-sedimentable DNA. The resulting virus was not precipitated by streptomycin.

It has been found that broth lysates are rich in desoxyribonuclease in contrast to F lysates which are very poor in this enzyme. It is considered that this coating of DNA is possibly removed from T2-N by the enzyme of the lysate. It is difficult to say to what extent these effects are due to decomposition products of T2*r*⁺ during preparation of lysates or of concentrates or are intrinsic qualities of the virus as reproduced.

The Isolation of the DNA of T2r^+ and T4r^+

I shall now leave the main theme of this paper, the metabolism of infected cells, to describe some unsuccessful ventures into the current fashion of nucleic acid biochemistry, that of inducing mutations or transformations. Since it had been shown by Hershey (1946) and by Delbrück and Bailey (1946) that a mixed multiple infection of *E. coli B* with T2r^+ and T4r produced T2r^+, T2r, T4r^+ and T4r, it seemed reasonable to attempt to effect the conversion of T4r within *E. coli* to T4r^+ by means of the DNA of T2r^+. Accordingly, it was necessary to isolate a polymeric and presumably "native" DNA from an r^+ virus. Since N viruses did not have the coating of DNA which appear to be irrelevant to properties of multiplication, the T2r^+-N and T4r^+-N preparations described in Table 1 were employed as follows: 3.6 gm. of urea were added to each 10 cc. of the virus preparation in 0.85% NaCl. The urea quickly dissolved with a volume increment at 37° of ca 25%. The viscosity of the solution at 37° at pH 8.3 rapidly rose to a maximum at ca 40 minutes. It remained essentially constant for at least 20 minutes. The values presented in Fig. 2 are corrected for the viscosity of urea and the volume increments. The very viscous solution was then deproteinized in the usual way with chloroform-caprylic alcohol (8:1). The protein-free supernate in the case of T2r^+ was approximately one-half as viscous as the original urea-disrupted virus. A fibrous polymeric salt of DNA from T2 was isolated by precipitation with alcohol in 98 and 96% yield with a P content of 9.3 per cent. The yield of nucleic acid from T4r^+ was only ca 60%. It may also be noted from the figure that the viscosity of irradiated inactive T2r^+-N in urea was only slightly less than that of fully active T2r^+-N.

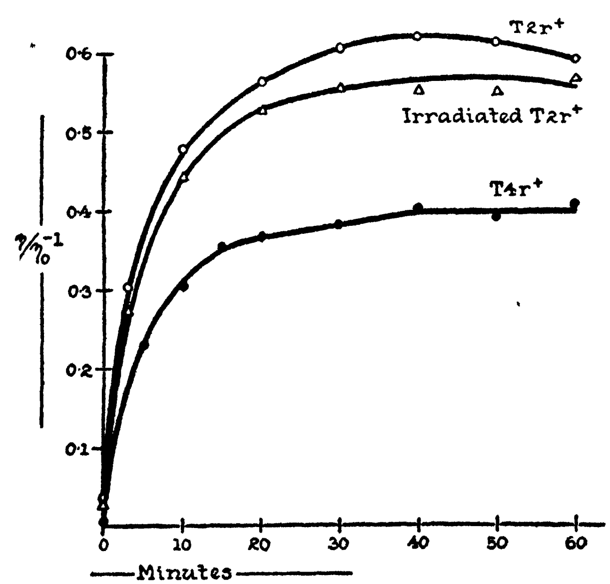

Fig. 2. The effect of the addition of urea on the viscosity of preparations of T2-N and T4-N.

It was found that the presence of DNA from irradiated T2 in cultures infected with T2 did not interfere with the multiplication of this virus. Nor did the DNA of active T2r^+ at a concentration of 50 γ per cc. induce mutations of T2r, T4r^+, or T4r within *E. coli.* It is possible that the polymeric nucleic acid of T2r^+ virus did not penetrate into the infected host. Hence the precise significance of these negative experiments is not clear.

These are the first "native" desoxyribonucleic acids isolated from viruses. Other isolations of DNA from viruses have depended on drastic degradations which affected the polymeric qualities of these materials. It is pertinent to point out that transformations with DNA among the viruses will depend, if at all possible, on isolation procedures which do not degrade the DNA.

The Multiplication and Respiration of Infected Bacteria

In most of the experiments which I shall describe, bacteria were grown in a synthetic medium F. containing inorganic P, lactate as a C source, and $NH4^+$ as a N source. They were multiply infected at an appropriate concentration with purified virus. At the concentration of infected cells employed, lysis inhibition occurred.

The effect of virus adsorption on the multiplication and respiration of the host has recently been reported (Cohen and Anderson, 1946). We found that the addition of T2 or T4 virus to bacteria growing in F medium in Warburg respirometers showed that concomitantly with infection a constant rate of O_2 consumption was established which was that of the uninfected bacteria just prior to infection. These effects are presented for a T4 system in Fig. 3. The turbidity of the culture changed only slightly during the interval presented. These facts signify that infection of the host cell inhibited multiplication of the host cell without change in the rate of O_2 consumption. The RQ of the infected bacteria was

also the same as of the uninfected culture.

It was also found that ultraviolet inactivated T2, which is unable to multiply within the host under conditions of single infection and prevents the multiplication of several other viruses within the host also stops bacterial multiplication without change in rate of O_2 consumption or RQ. Thus the active

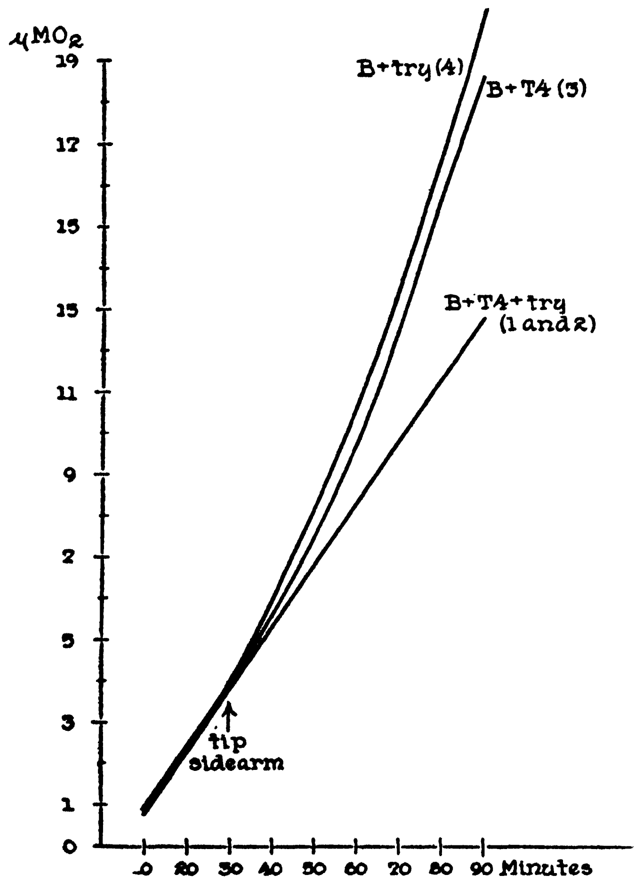

FIG. 3. The effect of T4r^+ virus adsorption on the rate of O_2 consumption of cultures of *E. coli B* in F medium. Try = tryptophane.

or inactive interfering viruses cause profound changes in the synthetic capabilities of the host without appearing to affect the energy supply of the cell. Monod (personal communication) has recently observed that infected cells also lose the power to adapt to the utilization of certain sugars, a characteristic property of uninfected *E. coli* multiplying in media depleted with respect to normally utilized sugars. This may be considered to be a confirmation of the totality of data presented in this paper stating that infected cells are incapable of enzyme synthesis.

This unequivocal result concerning the inhibition of cellular multiplication during virus infection was made possible by the technique of multiple infection described above. Under these conditions essentially all the cells of the system are infected. Contrary statements in the literature concerning cellular multiplication in bacteriophage systems, as well as in other virus systems, may be due to a lack of rigor in establishing the condition that the cell which is observed to multiply is indeed infected. This may conceivably be true even among the tumor virus systems, since it has been by no means ruled out that the stimulation of tumor multiplication may be due to a product other than virus, liberated from infected cells. In general, I know of no instance wherein it has been rigorously demonstrated that a cell in which virus is being multiplied is also multiplying.

SYNTHESIS OF DNA

In Fig. 4, it may be seen that in a culture of uninfected normal cells, the bacterial count, turbidity, and DNA content increased exponentially with time. An aliquot of this culture was infected with three particles of T2 per cell. As virus was adsorbed to the bacteria, the titer of infectious centers fell to the level of infected bacteria. Concomitantly the ability of the bacteria to multiply was lost, as revealed by the colony count and approximately constant turbidity. However, DNA was greatly increased. In fact the initial rate of synthesis of DNA per cell was ca 4 times as great in the infected culture as in the normal culture. Thus virus-infected cells have a stimulated rate of synthesis of this virus component. It will be seen subsequently that this apparent stimulation is due entirely to the transformation of the normal course of P assimilation exclusively to DNA synthesis.

The last statement anticipates the next aspect of our study, i.e., does RNA increase during infection by T2? In Fig. 5, it may be seen that RNA is constant in amount; it is not affected by the inhibition of DNA synthesis and virus multiplication produced by the action of the tryptophane antimetabolite 5-methyl tryptophane (Cohen and Anderson, 1946; Cohen and Fowler, 1947).

Ultraviolet-irradiated virus produces an effect in many ways similar to that of the analogue, 5-methyl tryptophane. After a 2-hour period, no significant increment in DNA or protein was noted. It may be hypothesized that the action of ultraviolet light has converted some portion of the virus, possibly DNA, into an analogue capable of blocking a vital enzyme site necessary for the synthesis of the virus DNA.

Another hypothesis might postulate that the synthesis of the components of a virus particle is a highly coordinated process depending on the presence of all template components. Thus destruction of some protein template by irradiation might inhibit the synthesis and accumulation of DNA. The fact that the efficiency of inactivation of T2

as a function of wave length approximates the absorption spectrum of DNA suggests the validity of the first hypothesis (Zelle, M., and Hollaender, A.—personal communication). The fact that DNA synthesis is indeed inhibited by the inhibition of protein synthesis, as by 5-methyl tryptophane, does not permit the second to be eliminated as yet. It will be quite important to make a choice between these hypotheses from the point of view of determining the site of action of ultraviolet irradiation and the site of the interference phenomenon in virus synthesis.

It appears, therefore, that although normal bacteria contain and, therefore, synthesize almost 3 times as much RNA as DNA, infected bacteria synthesized exclusively the type of nucleic acid characteristic of virus, namely DNA.

Assimilation in Infected Cells

What else is synthesized in these infected cells? We have confined our attention in the main to protein-bound constituents. Assimilation of N and P and synthesis of DNA were studied in the tri-

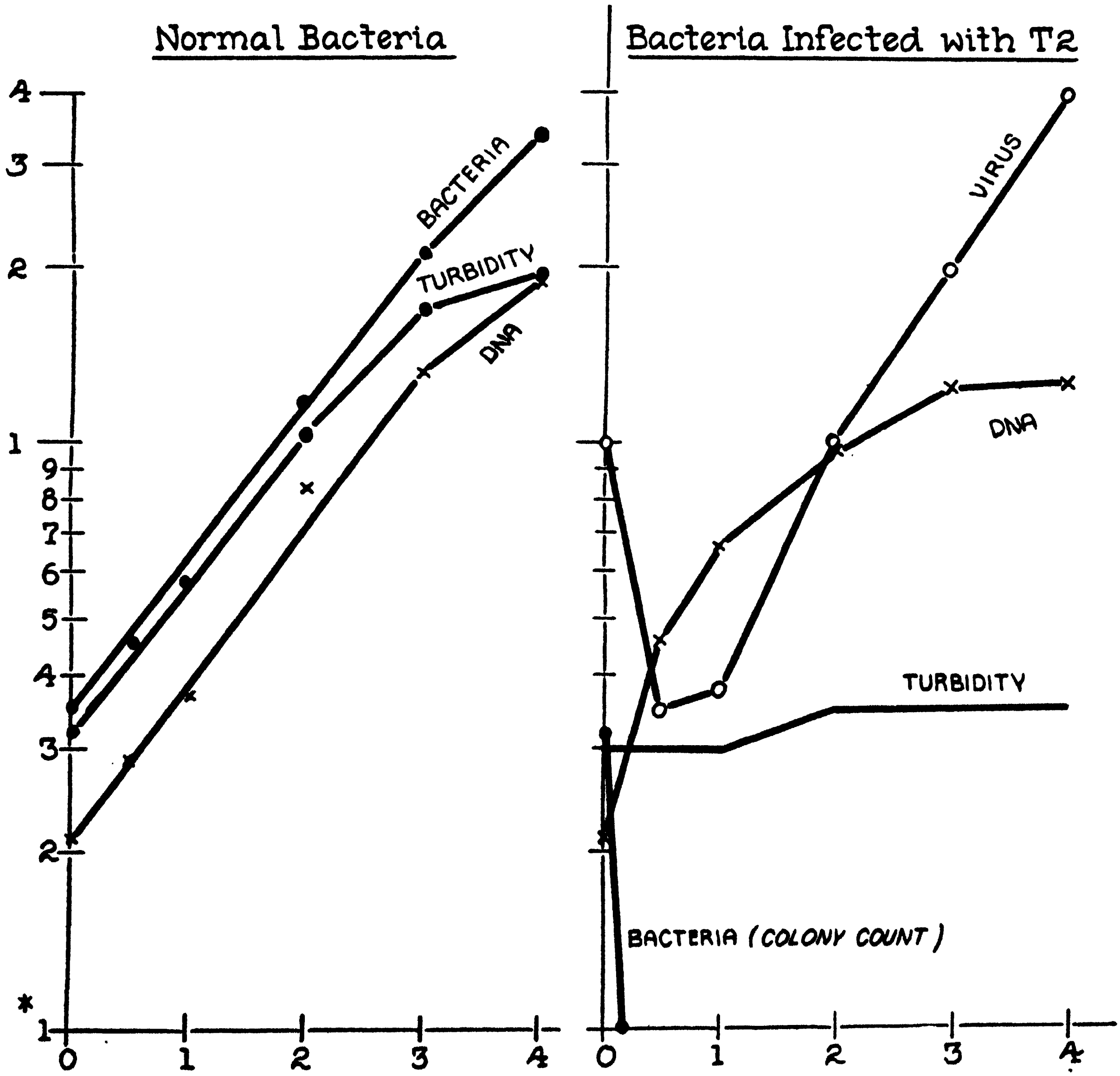

FIG. 4. Desoxyribose nucleic acid (DNA) synthesis in normal and T2r^+-infected *E. coli B* in F medium. *The ordinate represents 10^8 bacteria or virus per cc., 0.01 mg. DNA per 8 cc., or 10 units of turbidity.

chloroacetic acid precipitates (TCA) of normal and infected cells over a 2-hour period, as in Table 3. The bacteria start with a N/P ratio of 12.2, with DNA-P constituting one-fourth to one-fifth of the total protein-bound P, the rest being RNA, phospholipid, etc. After 2 hours of growth, in which time the bacteria have divided twice on this medium, the normal culture showed a considerable increment in these constituents, all of which are in approximately the same relation to each other as they were at the start of the experiment. In other words, normal bacteria synthesized normal bacterial components in the same ratio as they were present in the bacteria.

However, in the virus-infected cells, a very different situation prevailed. Far smaller amounts of N and P were added to the TCA-precipitable fraction. The increment in protein-bound P in infected cells is quantitatively accounted for by the increment in DNA. Thus DNA is the only protein-bound P-containing constituent which is synthesized by infected cells.

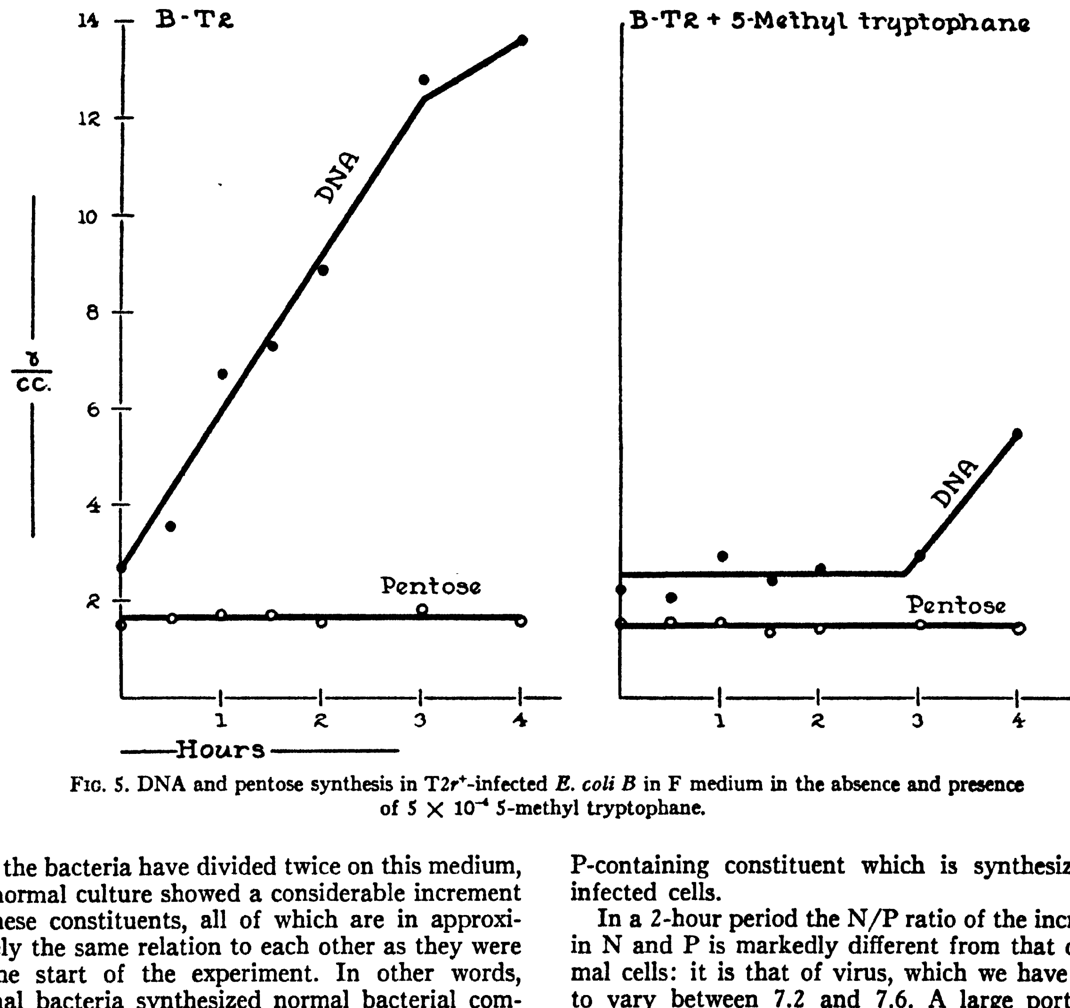

Fig. 5. DNA and pentose synthesis in T2r^+-infected *E. coli B* in F medium in the absence and presence of 5×10^{-4} 5-methyl tryptophane.

In a 2-hour period the N/P ratio of the increment in N and P is markedly different from that of normal cells: it is that of virus, which we have found to vary between 7.2 and 7.6. A large portion of these increments but by no means all can subsequently be isolated in virus. About one-fourth of

Table 3. Synthesis in Normal and T2-Infected *Escherichia coli B*

	mg. N/50 cc.	mg. P/50 cc.	mg. DNA/50 cc.	DNA−P / total P	N/P
B at 0 hours	0.333	0.0602	0.154	0.26	12.2
B at 2 hours	1.229	0.2219	0.422	0.19	12.2
$B_{2\,hrs.} - B_{0\,hrs.}$	0.896	0.1617	0.268	0.17	12.2
B−T2 at 0 hours	0.307	0.0685	0.179	—	—
B−T2 at 2 hours	0.430	0.1053	0.545	—	—
$(B-T2)_{2\,hrs.} - (B-T2)_{0\,hrs.}$	0.123	0.0368	0.366	0.99	7.38

the DNA, for instance, is found associated with bacterial debris after lysis. The relatively weak lysis in these r^+ systems, resulting in only partial disruption of the bacterial structures probably results in the retention of virus associated with bacterial structure in a manner analogous to the association of plant viruses with nonproteolysed cellular structures, described by Pirie (1946). However, in general at least three times as much DNA was isolated in the virus as was originally present in the cells which were infected.

Preliminary experiments in collaboration with Miss Catherine Fowler on carbon assimilation by normal and infected cells under conditions of limited F supply suggest that the ratio of lactate incorporated into body constituents to the percentage burned for energy is essentially the same in the two systems.

It appears then that after 2 hours of infection only the components of virus have been synthesized in protein-bound form, namely virus protein and one type of nucleic acid, in contrast to normal bacteria which synthesize many types of protein and enzymes, phospholipids, two types of nucleic acid, etc. This remarkable change in synthesis occurred without any apparent change in the energy supply provided by respiration.

The Origin of Virus Phosphorus

The results of these balance experiments contain the implication that the constituents of virus are derived from the constituents of the medium. It is conceivable, however, that the P-containing constituents of the normal bacteria yield their P to form virus DNA and that the normal constituents are resynthesized. This was tested by means of radioactive P in two ways.

Bacteria were grown in F media containing radioactive P, washed, and multiply infected in media free of radioactive P. It was found that the highest titer of virus appeared in ca 4 to 5 hours and the experiments were stopped at 5 hours. The single generation of T2 or T4 produced under these conditions was isolated, dialyzed, and analyzed. They contained little and, in one case, no radioactivity. In Table 4 are presented the results of an experiment in which the greatest amount of radioactivity appeared in T2 and T4. It may be seen that ca 82% of the P that appeared in the viruses was of non-radioactive origin and, therefore, came from the inorganic phosphate of the medium. It seems reasonable to suppose that the 5% of the total radioactivity of the bacterial culture which did appear in the virus may have been derived from the low-molecular P-containing metabolites present within the cell at the time of infection. These constituents then may have gone on to form virus DNA instead of bacterial constituents.

In the reverse type of experiment, non-radioactive bacteria were multiply infected in F media containing radioactive P. In a number of experiments the average radioactivity appeared in T2 or T4 virus to the extent of about 75% of the radioactivity of the inorganic P of the medium. From the results of the two types of experiment it appears therefore that the DNA of the virus is synthesized in the main from the inorganic phosphate of the medium after infection.

Table 4. Viruses Multiplied in Bacteria Containing Radioactive Phosphorus Suspended in Non-Radioactive Media

Fraction	mg P/cc.	mg DNA/cc.	Counts/min./10γP
E. coli B	0.0144	ca .024	293
$T2r^+$	0.0100	0.100	53.5
$T4r^+$	0.0104	0.110	53.3

Is RNA a Precursor of DNA?

One finds much discussion of this hypothesis and the data on which it is based are ably summarized by Brachet (1944). Several more or less indirect types of evidence bear on this point, that derived from ultraviolet microscopy being of the more indirect type. The data of Brachet (1937) on the conversion of ribose nucleotides to DNA nucleotides in the early stages of cleavage of the fertilized echinoderm egg are the strongest evidence for the hypothesis.

It is necessary to state that the hypothesis of the transformation of intact RNA to DNA involving only a methylation of uracil and a reduction of the C_2OH of ribose appears improbable from considerations of the structures of RNA and DNA, as reviewed by Tipson and in the symposium by Gulland (1945).

The system which we are employing is one which seems to permit a definitive test of the hypothesis. The previous data strongly suggest that a conversion of RNA nucleotides to DNA nucleotides is unlikely since RNA remained constant in amount during infection. Nevertheless, a turnover in RNA has not been ruled out. Accordingly, the turnover of RNA-P was followed during infection by means of radioactive P.

E. coli B was infected in the presence of radioactive P. After one hour, the RNA and DNA fractions were quantitatively isolated from the infected cells by the method of Schmidt and Thannhauser (1945). It was found in several experiments that in this time the increment in protein-bound P was essentially the same as the newly synthesized DNA-P. The radioactivity per γ increment in P in the bacteria was the same as that of this value in the medium.

In the experiment presented in Table 5, the final amount of DNA was 3.0 times the initial DNA.

Hence, the radioactivity of this fraction was corrected for the presence of the original bacterial DNA. The fraction of newly synthesized DNA then had a radioactivity essentially identical with that of the inorganic P of the medium. On the other hand, the RNA after correction for traces of DNA, as determined by the diphenylamine reaction (Dische, 1930) had practically no radioactivity. The RNA-P fraction was quantitatively accounted for by its pentose-3 phosphate content. Hence in this system

TABLE 5. INCLUSION OF RADIOACTIVE P INTO NUCLEIC ACIDS OF *E. coli B* INFECTED WITH $T2r^+$ BACTERIOPHAGE

Nucleic acid	% total protein-bound P	% lipid-free bacterial residue	Counts/ γP/min.	Corrected Counts/ γP/min.
DNA	48	13.8	60	90
RNA	36.5	10.5	8.8	2

DNA—Counts corrected for presence of original bacterial DNA.
RNA—Counts corrected for presence of traces of DNA in RNA fraction.
Counts/γP/min. of medium = 93.

RNA has no significant turnover, is probably not a precursor of DNA, and appears essentially inert after infection.

That there are precursors to protein-bound DNA from ammonia, lactate and inorganic phosphate cannot be questioned and they must be looked for in the protein-free soluble fractions. This appears to be the most important problem of nucleic acid metabolism at the present time. We have made some preliminary experiments in this direction with radioactive P in the *E. coli*-T2 system without marked success. The amounts of organic P in the TCA-soluble, Ca-soluble, Et OH precipitable fraction are minute. Nevertheless, we have found that some 75% of the organic-P in this fraction is in the form of ribose-5-phosphate (Albaum and Umbreit, 1947). Radioactivity was also found in this fraction but in amounts such that it was not possible to say that the ribose-5-phosphate P contained radioactivity.

It is possible that this can be worked out with very large amounts of infected bacteria. The one-way attributes of this system are favorable. Nutritional studies carried out in collaboration with Miss Fowler have revealed methods of testing the efficacy of certain intermediates for virus synthesis (Cohen and Anderson, 1946; Cohen and Fowler, 1947; Fowler and Cohen, 1947). It has been observed for instance that guanosine and desoxyguanosine markedly stimulate virus multiplication far more than guanine alone. The conversion of the former to ribose-1-phosphate (Kalckar, 1947) and probably ribose-5-phosphate (Schlenck and Waldvogel, 1947) suggest numerous possibilities for the formation of desoxyribonucleotides in this system.

THE RATES OF DNA AND PROTEIN SYNTHESIS IN INFECTED CELLS

According to Table 3, and subsequent data, the end products of assimilation in the *E. coli*-T2 system appear in material having the general composition of virus and actually may be isolated in large part as virus. If the virus particles contained the

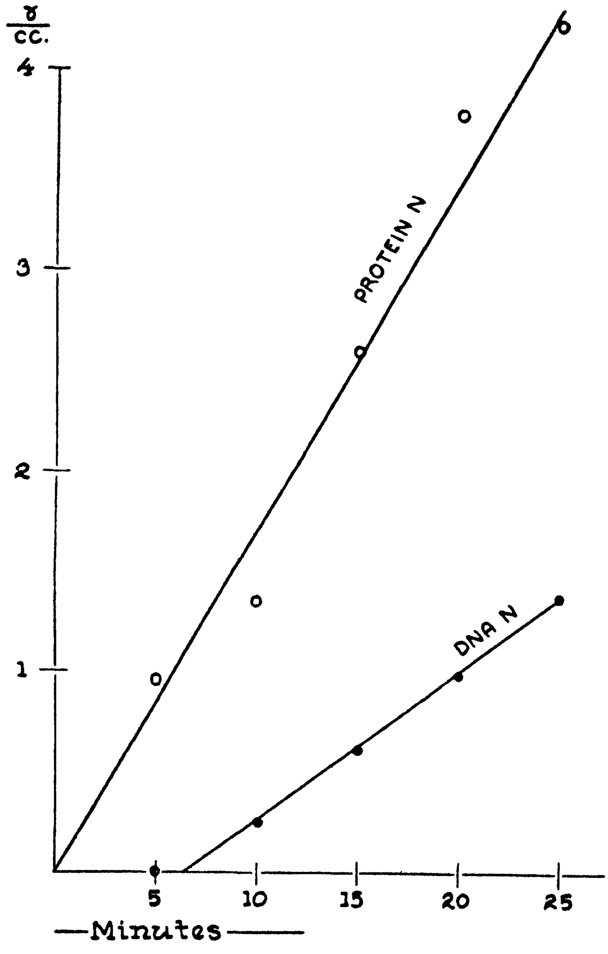

FIG. 6. The synthesis of protein and DNA in $T2r^+$-infected *E. coli B* in F medium prior to establishment of lysis inhibition. The ordinate presents increments over initial values.

enzymes for the synthesis of DNA and protein, it might be expected that as virus multiplied within the cell, these constitutents would be synthesized at an increasing rate, assuming that the precursors of DNA are supplied in maximal amounts. It was noted in Fig. 4 that in uninfected cells DNA actually increased at a logarithmic rate.

It was found, however, as seen in Fig. 6, that the

rates of increment of protein and DNA were constant in the period prior to the establishment of lysis-inhibition. Protein is synthesized from the moment of infection while DNA synthesis did not begin until a short latent period was completed. It has been found that the burst size in F between 25 and 30 minutes is ca 20 to 30. The most active T2 we have obtained had 1.6×10^9 infectious units per γ DNA. In five experiments of this type under comparable conditions, 2.2 to 2.6 γ DNA were synthesized by 2×10^8 cells in 25 minutes, yielding an equivalent of 17 to 20 T2 particles produced in up in virus. It will be necessary to devise some method of breaking open infected cells and simultaneously following the increase in virus activity as well as virus constituents. It is conceivable, although unlikely, that the rate of interaction of the components to form virus will be different from the rate of synthesis of the slowest component, DNA.

It may be calculated from the data in Fig. 6 that the atomic ratio of the rates of increment of total N to P is 12.4, which is that of the ratio of the assimilation of N and P in normal bacteria. An

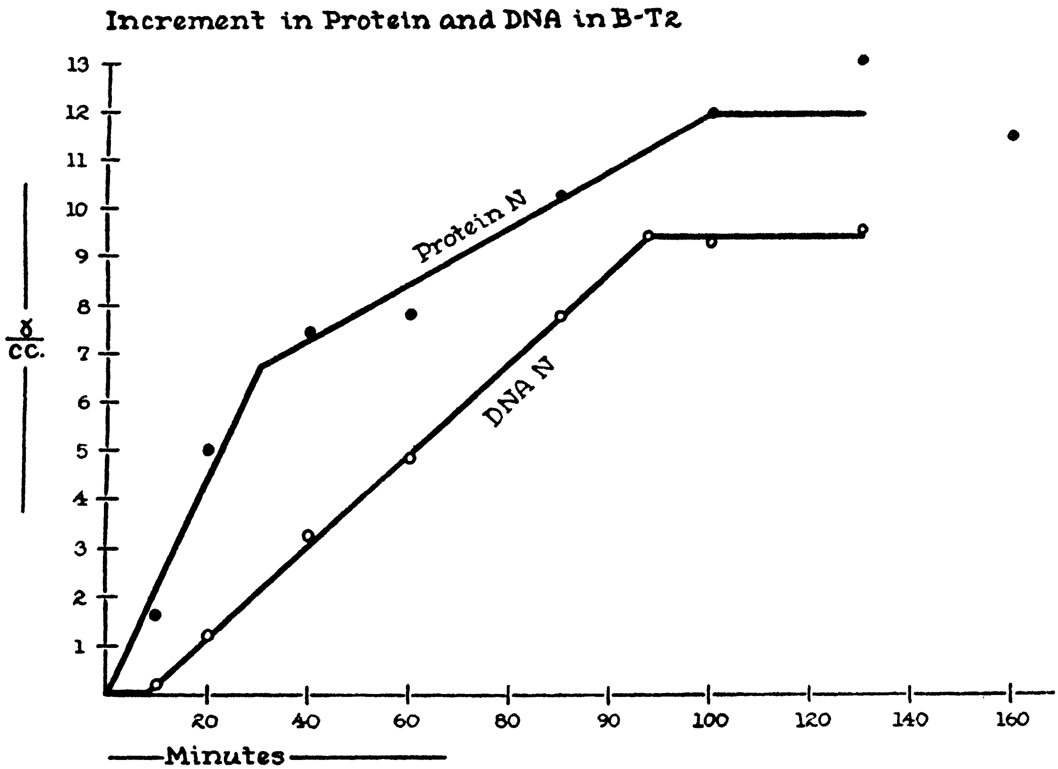

FIG. 7. The synthesis of protein and DNA in T2r^+-infected *E. coli B* before and after establishment of lysis inhibition.

this time. It is likely that this value should be corrected upwards since our most active T2 undoubtedly contains a significant proportion of inactive virus. Nevertheless this approximation suggests that DNA synthesis is a close approximation of virus synthesis.

Since the rates of synthesis of these components are linear, this may be taken as evidence that the virus is not the center of DNA or protein synthesis and that new centers have not been synthesized or if synthesized are not being used for virus synthesis. It is clear that this is only an indirect approach to the problem which so far seems consistent with the observed phenomena. What has been measured are the rates of synthesis of two components which turn apparent contradiction in the data appears, at this point, since it was shown previously that in a 2-hour interval, the end-product was material of the constitution of virus, having an N/P of 7.4, while the constant rates of increment of N and P had a ratio of 12.4, that of the bacteria. This has perhaps been resolved by the observation that in most instances (one exception was noted in 5 experiments) the rate of protein synthesis was sharply reduced between 30 and 50 minutes while the rate of DNA synthesis was unchanged, as in Fig. 7. After 30 minutes, the ratio of N to P continually decreased until it approached that of virus, at which point synthesis of DNA abruptly stopped. This point which generally appears at ca 2 hours, is also

marked by the beginning of the liberation of virus and proteolysis, as measured by a rapid decrease in TCA-precipitable N. It appeared therefore that the excess of protein synthesized in the first 30 minutes was then depleted to form virus in the ensuing period.

A tentative hypothesis has been formulated suggesting that the break in the rate of protein synthesis, coming at the approximate time of the establishment of lysis-inhibition, is due to that phenomenon. If true, the hereditary factor, r^+, would have two manifestations, one involving lysis inhibition, and the second the inhibition of protein synthesis, possibly centering in its action on an enzyme capable of both proteogenesis and proteolysis, the latter being known to occur during lysis.

Doermann appears to have shown that burst sizes in lysis-inhibited systems increase with the increase in the total latent periods, indicating that virus multiplication is still unimpaired. The increases in burst size were roughly proportional to the increase in latent period, suggesting the linear synthesis of virus during lysis-inhibition. Thus, the observed linear synthesis of DNA throughout this period is quite consistent with his observations, and is another point in favor of the hypothesis that the rate of formation of protein-bound DNA is a measure of the rate of synthesis of virus.

The Latent Period of DNA Synthesis

As described in Figs. 6 and 7, DNA does not increase for 8 to 10 minutes after infection. Four lines of evidence suggest that this represents the time necessary for the synthesis of virus peptides essential for the reception and organization of newly synthesized virus nucleotides. These are:

1. Protein is synthesized from the beginning of infection. That this is indeed virus protein is suggested by the viral ratio of N to P at the end of the lysis-inhibited multiplication, and the approximate equivalence of virus yield and DNA synthesized. It is apparent from the curves presented that at the beginning of DNA synthesis there is protein without nucleic acid, and that at 30 minutes there is a considerable excess of protein free of virus nucleic acid. Whether this excess protein is indeed virus protein is presumably subject to test, perhaps by immunochemical methods.
2. DNA synthesis stopped when the excess of protein was markedly depleted and the final ratio approached that of virus.
3. 5-methyl tryptophane which appears to compete specifically with tryptophane prevented DNA synthesis.
4. It is possible to reduce the latent period of DNA synthesis and increase the rate of synthesis of DNA by increasing the rate of peptide synthesis. This is most clearly demonstrated in a system in which the latent period was unusually prolonged by the following method: *E. coli B* was grown in broth, instead of F. When this organism was transferred to F and washed in F its synthetic abilities were much slower. Under these conditions, infection in F resulted in an extended latent period of virus multiplication and DNA synthesis, with a low yield of virus, as compared to these functions in broth.

The supplementation of F by single components only partially made up the difference in virus latent

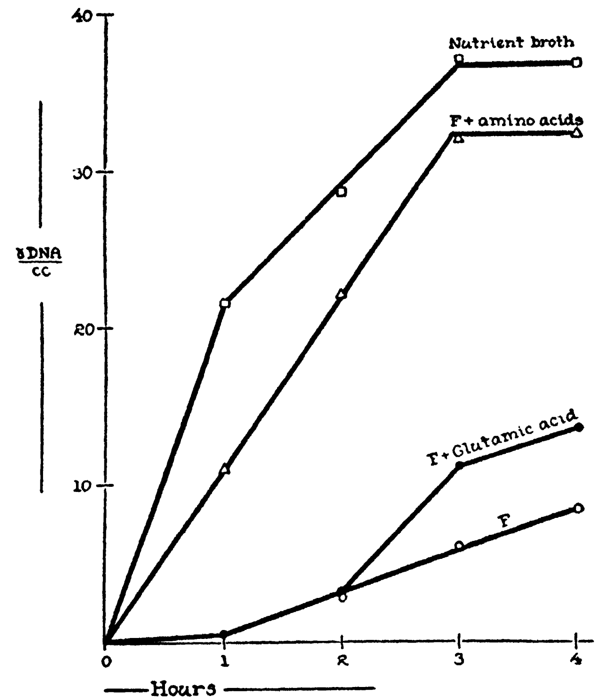

FIG. 8. The synthesis of DNA in T2r^+-infected *E. coli B*. The bacteria were grown in nutrient broth, washed, and suspended in various media. The ordinate presents the increments over the initial values.

period. Glutamic acid was the most potent of the amino acids in this respect. A casein acid hydrolysate plus tryptophane added to F almost, but not quite, duplicated the latent period of virus multiplication and the burst size. Virus production in F plus a casein hydrolysate plus tryptophane, plus a mixture of purines and pyrimidines appeared to approximate more closely the production of virus in broth.

In Figure 8, we have followed DNA synthesis under the conditions described above. It may be seen that the rate of DNA synthesis was markedly stimulated by amino acids and that the latent period was essentially eliminated.

Discussion

I wish to call your attention to a paper by Gratia, Jeener and Brachet (1945) in which cytochemical

studies are presented on silk worms infected by the virus of silk worm jaundice. The non-multiplying infected cells had no change in the rate of O_2 consumption and rapidly synthesized the DNA that appeared in the virus inclusion bodies. In this study, only a single major difference from our own results appears. RNA seems to be metabolizing and increasing at a great rate although it is probably not a virus constituent. Thus the results of this study seem to emphasize numerous similarities of very different virus infections and simultaneously open up the possibility of entirely new relationships. This is the only other comparable study in the literature and reveals cytochemistry as a very powerful tool in virology. It is unfortunate that cytochemical techniques for bacteria are not as useful as for larger cells.

In view of the recent generalized treatment of the problem of specific protein synthesis by Spiegelman and Kamen (1946), which was an extension of statements of Caspersson and Brandt (1940) on this question, it is pertinent to consider some of their hypotheses at this point.

They have proposed that nucleoproteins (*e.g.*, T2) are controlling elements in protein synthesis since they supply the necessary energy for synthesis in the molecular structure of one of the reactants, *i.e.*, nucleic acid. Thus: "We may further plausibly suggest that these phosphorus-containing proteins are the specific energy donators which make possible reactions leading to protein and enzyme synthesis." And, self-duplicating units are "likely to possess the ability to transform and accumulate energy within their own molecular structure which can be used for the synthesis of similar units. At any rate, it is relatively easy to show that the growth kinetics of such 'energy accumulators' is that of the self-duplicating or autocatalytic type."

Now, in view of the fact that our data indicate that the energy and substance for virus synthesis are supplied in entirely normal fashion by the parasitized cell without the participation of RNA, and that the kinetics of synthesis of virus components is not of the S-shaped type, it seems unnecessary to attempt to apply these hypotheses to our system at this time. One readily apparent mechanism for virus synthesis is the time-worn template hypothesis, since virus does not seem to be a self-duplicating unit at all but rather a duplicated unit. Thus the virus appears to be synthesized by the cell according to the models (templates) which it provides for the host's enzymes.

This hypothesis although simple to formulate is far from simple to understand and still less so to test in this system. Considering the present state of knowledge of synthetic mechanisms for the nucleic acids and proteins, it appears probable that it will take a very considerable period indeed to understand the nature of template action. We may consider ourselves fortunate to be able to refer at this point to the increasing knowledge of the mechanism of specific polysaccharide synthesis (glycogen, starch) for the assurance that we may eventually approach our problem directly.

In addition to the conclusion that our virus is synthesized by the host enzymes, our data strongly indicate that virus components are derived largely from the external environment. This has been most conclusively determined for P but the interrelations of utilization of the other constituents imply its validity for carbon and nitrogen as well. Isotope techniques with these elements will probably establish this also. Thus, these data appear to be disastrous for the virus precursor hypothesis, at least in this system. This hypothesis proposes that precursor (proteinogen) exists in the cell prior to infection and is autocatalytically transformed to virus by infecting virus. There is a modification of the hypothesis which assumes the presence of a trace of precursor proteinogen which is then synthesized in increasing amount after infection as the original stock proteinogen is converted to virus. Since the trace hypothesis is not susceptible to experimental test it need not be further discussed.

In so far as the results obtained on the synthesis of virus components in this system fit so well with the starting hypothesis, derived only from considerations of the composition and activities of a great many different viruses, it would appear possible to suggest a generalization which is subject to test. The generalization takes the form of a definition of "virus": a virus is a parasite which organizes a specific enzymatic environment for its own multiplication. This is in contrast to other forms, such as bacteria, which organize a simpler environment with their own enzymes, or a gene which is generally not considered to be parasitic. It does not eliminate the possibility that vaccinia, for instance, containing flavin-adenine dinucleotide may be found to possess a few enzyme systems and so may constitute an intermediate form if it may be demonstrated that those potentially existent enzyme systems are indeed essential for multiplication. Thus the application of the definition may permit a functional classification of these lower forms.

Thus the most important test of the generalized hypothesis requires the extension of this type of study to other systems. It seems to me that this type of study in influenza virus systems, for instance, holds the key to some pressing practical problems, such as the control of influenza virus multiplication. It also presents an approach to the possible exception in virus composition which has been reported (Knight, 1947).

Preparations of influenza virus appear to contain particles bearing both virus activity and antigens of the microsomes of the host cell. If short range forces are assumed to be sufficient for virus synthesis by the template method, it would be necessary that the specific structures characteristic

of virus become closely associated with the enzymatic centers of synthesis. It has been demonstrated that microsomes are indeed rich in enzymatic activity and thus a union of the two structures is not unreasonable. It would appear to be one characteristic of this group of viruses that dissociation of the complex is not readily attained, resulting in virus preparations containing antigens characteristic of the host in which the virus has grown.

Knight (1947) has reported the presence of both RNA and DNA in these viruses. Since microsomes are rich in RNA, it is possible that the RNA of influenza virus preparations is not synthesized after infection but merely transferred while DNA is indeed a synthesized constituent. If this hypothesis is true and it can be tested by means of radioactive P in the manner which has been described, only the DNA would be considered to be the virus nucleic acid since it would be the nucleic acid which participates in the organization of a specific enzymatic environment for its own multiplication.

Another perspective implicit in the definition postulates the synthesis of virus in an enzymatic environment without recourse to cells capable of normal growth and division. Of course, infected cells are incapable of normal growth and division and cells rendered incapable of mutiplying by means of irradiation (Anderson, 1944; Rouyer and Latarjet, 1946) still support virus multiplication. What is, in fact, proposed is the isolation of and appropriate arrangement of the enzymes involved in a suitable mixture of metabolites, provision of a suitable energy source, the addition of some virus template, and collection of the newly synthesized virus. It can not be considered that this is beyond the possible achievements of present day biochemistry. With respect to this last project, one may distinguish sharply between performance and understanding.

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DISCUSSION

SPIEGELMAN: There are certain aspects of Dr. Cohen's interesting and suggestive paper which I should like to discuss from the point of view of the general problem of self-duplication. The fact that a straight line was obtained in the DNA synthesis cannot be taken as evidence against the existence of a self-duplicating process. The exponential nature of the autocatalytic curve is only expressed at the onset when all resistances, both environmental and competitive, are at their minimum. As soon as these factors become determining the curve ceases to be exponential and can take on almost any shape, including a linear one. In this connection it must be noted that the burst size (the number of individuals formed in each bacterium) is of the order of 100, which corresponds only to about 7 generations. It is perhaps not surprising therefore that little if any of the exponential portion of the curve was realized.

From the point of view of the general biologists the distinction drawn by Dr. Cohen between a "self-duplicating" and a "self-duplicated" unit may prove to be somewhat confusing. It is customary to classify a unit as self-duplicating if it satisfies both of the following criteria:

(1) At least one of the units is required for the formation of others.

(2) The unit should be capable of undergoing modifications (mutations) which are capable of further duplication.

Since viruses satisfy both of these criteria, they should be classified as self-duplicating units. What Dr. Cohen wishes to emphasize is fundamentally a problem in metabolic requirements of one particular class of self-duplicating units. This is certainly a legitimate emphasis but perhaps can be done without tampering with the basic definition of "self-duplication."

Finally I should like to enter a protest against the use of the word "template," which is often employed in discussing the mechanism of self-duplication. Dr. Cohen of course recognizes that it is impossible to define or understand what this word actually signifies. The difficulty with using the word template is that it unfortunately generates a false air of understanding. The only thing it actually implies is the first part of the definition of self-duplication, *i.e.*, one is needed to get another. It does not in any way provide a mechanism whereby the first yields the second. Its use, therefore, has little explanatory value in so far as the mechanism of self-duplication is concerned and has been in the past relatively sterile in stimulating an experimental approach to this problem.

COHEN: There are two reasons why I feel obliged to tilt at "self-duplicating." The first, and possibly less important, is that the most obvious picture of virus synthesis deduced from my data has the infecting virus particle passively supplying models to the host's enzymes for the conversion of substrate to more virus. Although "self-duplicating" seems to carry no implications of mechanism to many workers, the term does not possess that neutral shade to me.

The more important reason is the fact that the use of the term seems to relegate the environment to a back-seat role. I consider it significant that the criteria for "self-duplication" proposed by Dr. Spiegelman do not include the word "environment." It is significant in a practical sense because in the course of much experimentation to establish the minimal chemical characteristics of a "self-duplicating" unit, there has been virtually no biochemical study of the relation of the virus to its host, to its environment. The term has been a theoretical fortification for the methodology of organic chemistry which has been almost exclusively applied to the chemical study of the viruses (*i.e.*, isolation and characterization).

As far as virus multiplication is concerned, I consider an exact description of the minimal environment, and of the interaction of the virus with its environment, to be as important as an exact description of a particular virus. It seems to be time that we began to learn whether there is indeed a difference between a bacterium which unquestionably divides to form two and handles its environment with its own enzymes and a virus which may or may not do these things. I think there is a large difference is quality between a form such as *E. coli B*, which uses its own enzymes on a relatively simple environment at one end of the scale, and $T2r^{+}$ bacteriophage, which seems to use the enzymes of *E. coli B* as its environment. That there are intermediate forms also, I do not doubt, but I wish to know which they are and in what respects they are intermediate. These will not be defined only in terms of the unit but will require a description of a rela-

tion of unit to environment. I dislike the use of "self-duplicating" because, by resulting in the development of one-sided hypotheses and approaches, it tends to interfere with the search for specific mechanisms of reproduction.

DOYLE: The point raised by Spiegelman with respect to a linear increase in DNA along with reproduction of the self-duplicating nucleus presents no serious criticism unless the nucleus doubles in mass for each division. Thorell pointed out that in maturing blood cells there is multiplication of nuclei with no increase in desoxyribonucleic acid.

BRACHET: Concerning the nonconversion of RNA into DNA during phage multiplication, it should be noted that this system is very different from the case of the sea urchin's egg, where no intake of phosphate from the outside is necessary during early development. In the phage, on the contrary, most of the phosphate required for DNA synthesis comes from the outer medium. It is well conceivable that entirely different mechanisms are at work in both cases.

RIS: From the table of Dr. Cohen it appears as if the Rickettsiae did not contain any ribonucleic acid. It seemed possible to us that the ribonucleic acid was removed during the preparation of the material which Dr. Cohen studied, since it is well known that ribonucleic acid is very easily washed out of cells even in physiological saline. To check on this, we smeared fresh Rickettsiae and material washed with physiological saline on the same slide, fixed with acetic-alcohol and stained with pyronin. The unwashed organisms stained heavily with pyronin while the washed ones stained hardly at all. Since under the conditions used pyronin appears to be specific for ribonucleic acid we must conclude that most likely Rickettsiae contain not only desoxyribonucleic acid but also ribonucleic acid which is easily removed during preparation for chemical analysis.

H. TAYLOR: Dr. Cohen implies that in measuring DNA changes in the infected bacterium he is thus measuring virus multiplication. Much of his interpretation rests upon this assumption, and its validity should be critically examined. In the early portion of his report, Dr. Cohen discusses the preparation and properties of purified virus obtained from lysates. He has made a point of the observation that phage liberated in synthetic medium differs in size and physical properties from that obtained in broth lysates. By treating with desoxyribonuclease, the purified virus obtained from lysates in synthetic medium can be divested of a portion of its DNA without reducing its infectivity. Since this is so, one might question the correctness of assuming that the total DNA of the infected cell is going to be included in the virus particles. I believe that in studies of bacterial growth, for example, whenever anything other than cell count has been used as a measure of population density, proof has first been obtained that the substance or property measured bears a known relationship to the increase in number of organisms. The establishment of such a relationship between measured DNA and infection units seems unproven as yet. This matter also seems quite separate from the question of whether the DNA and protein is of the kind used by the bacterium or the virus. It seems quite possible, for example, to visualize the protein and DNA synthesized by the infected cell as a substratum for virus formation, the synthesis of which is initiated by the infecting particle. The substratum may be synthesized in excess of the amount actually needed for formation of the new virus particles.

COHEN: I'm afraid that Dr. Taylor considers my hypothesis (that the rate of DNA synthesis is the rate of virus synthesis) to be my conclusion. I have indicated some data in support of the hypothesis, but explicitly stated that its proof would depend on a direct correlation of virus count within the infected cell and the increment in DNA. It has not yet been possible to make an estimate of the number of virus particles within infected *E. coli*, and in view of this serious experimental difficulty, one can only hypothesize concerning the significance of the finding that the virus component, DNA, is synthesized at a constant rate.

Nevertheless, it seems pertinent to summarize the evidence in support of the hypothesis:

(1) We found protein-bound DNA synthesis to begin at 7 to 10 minutes after infection. Luria and Latarjet found the ultraviolet inactivation curve of single-infected cells to be of the single-hit type until 7 to 8 minutes, when the form changed.

(2) After 25 minutes of infection in F medium, the increment in DNA is equivalent to an amount of T2-F which approximates the actual burst size in F at about that time.

(3) The rate of DNA synthesis is constant from 10 to about 120 minutes. Doermann's data indicate a constant rate of virus synthesis in lysis-inhibited cells, which in our system would include the interval of about 35 to 120 minutes.

Thus the origin and several later points on the curve of virus synthesis seem to be accounted for by the curve of DNA synthesis.

SOME FACTORS INFLUENCING THE NUCLEIC ACID CONTENT OF CELLS AND TISSUES

J. N. DAVIDSON

Our views on the distribution of the nucleic acids have undergone profound changes since Jones (1920) stated that "there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells and the other from the nuclei of plant cells." During the last 25 years evidence has gradually accumulated to show that such a distinction between the two main types of nucleic acid is not valid and that both types of nucleic acid occur both in plant cells and in animal cells. This subject has already been reviewed (Davidson and Waymouth, 1944e).

Evidence that compounds containing pentose sugars are present in animal tissues has been available since the beginning of the century. Such compounds include of course the simple mononucleotides such as adenylic acid, but the quantity of pentose-containing derivatives in many tissues is far greater than can be accounted for by the presence of simple nucleotides, and the occurrence of pentose polynucleotides in animal tissues was confirmed by the isolation by Jorpes (1924, 1928b, 1934) of a pentose polynucleotide from the pancreas. Since then, pentose polynucleotides ("ribonucleic acids") have been isolated from a number of other animal tissues, including liver, kidney, intestine, thymus, and even from the larvae of Calliphora (Grégoire, 1944). Moreover, the early work of Caspersson and Schultz (1938, 1939, 1940) revealed that the cytoplasm of many rapidly growing cells contained a high concentration of material which, on the basis of strong absorption of ultraviolet light at 257 mμ and a negative Feulgen reaction was assumed to be ribonucleic acid. It appeared therefore that not only was ribonucleic acid to be found in animal tissues but that it was particularly abundant in rapidly proliferating tissues, and at this stage it obviously became desirable that analytical data for the ribonucleic acid and desoxyribonucleic acid content of different tissues should be available. Jorpes (1928a) had published figures for the total nucleic acid phosphorus and for the pentose content of a number of tissues (pancreas, liver, spleen, thymus, parotid, stomach) and in 1941 Brachet published a similar series of figures for the pentose content of a large number of tissues of the rabbit and the frog. The estimations made by Jorpes were carried out on fresh tissue and therefore gave only approximate values for the ribonucleic acid content since fresh tissue may contain small amounts of pentose derivatives other than polynucleotides and since, in any case, pentose estimations are liable to be upset by a number of interfering factors, *e.g.*, uronic acids.

An attempt was made to overcome these difficulties in a systematic investigation made by Davidson and Waymouth (1943a, 1944a) on the tissues of the adult and embryo sheep. This species was chosen because most tissues, even those of the embryo, could be obtained in fairly large amounts. The fresh tissue was exhaustively extracted both with lipid solvents and with acid so as to remove lipid phosphorus and acid soluble phosphorus compounds. The protein-bound phosphorus left in the residue after such extraction is derived almost exclusively from nucleic acids, but includes also the very small amounts of phosphoprotein phosphorus present in most animal tissues. The tissue powder was treated first with cold and then with hot 10% sodium chloride solution so as to extract the nucleic acids. Concentrated sodium chloride solutions may conveniently be used in this way to extract nucleic acids from a tissue powder containing denatured protein without at the same time removing very large amounts of protein. When used with fresh tissue the effect of 10% sodium chloride is of course to bring the desoxyribonucleohistone into solution (Mirsky and Pollister, 1946).

From the combined extracts the nucleic acids were precipitated as the lanthanum salts which were submitted to analysis for pentose and desoxypentose by standard colorimetric methods using calibration curves prepared from purified yeast ribonucleic acid and purified thymus desoxyribonucleic acid respectively, in order that the nucleic acid content of the unknown could be expressed directly in terms of phosphorus. This procedure involves the assumption that the relative amounts of pentose, or of furfural yielding material, to phosphorus, are the same in nucleic acids from all organs, and that the relative amount of reactive desoxypentose to phosphorus is the same in the desoxyribonucleic acids from all tissues. These assumptions may not be justifiable for all tissues, but since it has been shown that liver ribonucleic acid has the same pentose content relative to phosphorus as has yeast ribonucleic acid, the method appears to be justified in the case of liver tissue which has been most commonly employed in our work. In most other tissues it has been found possible to account for nearly all of the residual phosphorus in terms of either ribonucleic acid or desoxyribonucleic acid.

This original method is tedious to use and is not capable of yielding very accurate results, partly because the extraction of nucleic acids with sodium chloride is not quantitative. In an effort to overcome this difficulty Euler and Hahn (1946) have recently described a modified procedure in which very dilute alkali is used to complete the extraction. The original procedure has nevertheless proved valuable in giving an estimate of the relative amounts of the two types of nucleic acid in a large series of tissues, and it has revealed that in many tissues the amount of ribonucleic acid is surprisingly high and may greatly exceed the amount of desoxyribonucleic acid. In some tissues such as liver, it was subsequently found possible to carry out pentose and desoxypentose estimations directly on the extracted tissue powder, but in other tissues, interfering factors tended to reduce the value of such direct estimations.

Schneider in 1945 developed a method of treat-

TABLE 1. A COMPARISON OF THE RESULTS OBTAINED BY DIFFERENT AUTHORS FOR THE NUCLEIC ACID CONTENT OF TISSUES, EXPRESSED AS MG. RIBONUCLEIC ACID PHOSPHORUS (RNAP) AND MG. DESOXYRIBONUCLEIC ACID PHOSPHORUS (DNAP) PER 100 G. FRESH TISSUE

Tissue	Species	RNAP	DNAP	Ratio R/D	Method	Author
Liver	Ox	70	34	2.1	Calculated from pentose estimations.	Jorpes (1928a)
	Rabbit	57	19	3.0	Calculated from pentose estimations.	Jorpes (1928a)
	Rabbit	44	—	—	Calculated from pentose estimations.	Brachet (1941a)
	Sheep	—	—	3.5	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Rat	101	24	4.3	Pentose and desoxypentose estimated on extracted tissue.	Davidson and Waymouth (1944c)
	Rat	64–100	20–22	3.9	Calcualted from pentose and desoxypentose estimations.	Schneider (1945)
	Rat	87–102	22.5–26	3.3–4.6	P estimations	Schmidt and Thannhauser (1945)
	Rat	55–72	21–31	2.5	From pentose and desoxypentose estimations.	Schneider and Klug (1946)
	Rat	77.7±4.1	26.0±1.5	3.0	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
	Rat	68–99	27	3.7	Calculated from pentose and desoxypentose estimated on La salts.	Euler and Hahn (1946)
	Rat	69	36	1.9	Calculated.	Hammarsten and Hevesy (1946)
	Rat (200–240 g.)	77–110	21–25	4.0	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rat (60–80 g.)	106–122	28–37	3.6	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rat (pregnant) female	110–118	21–23	5.2	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rat (embryo)	87–134	35–65	2.2	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rabbit	44–76	16–29	2.7	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rabbit (pregnant female)	67–138	14–17	6.8	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rabbit (embryo)	87–105	61–84	1.3	Method of Schmidt and Thannhauser.	Davidson (1947)
	Cat	72–85	25–43	2.3	Method of Schmidt and Thannhauser.	Davidson (1947)
	Sheep	55–84	23–33	2.5	Method of Schmidt and Thannhauser.	Davidson (1947)
	Man (one sample)	37	19	2.0	Method of Schmidt and Thannhauser.	Davidson (1947)
Pancreas	Ox	188	35	5.4	Calculated from pentose estimations.	Jorpes (1928a)
	Rabbit	140	—	—	Calculated from pentose estimations.	Brachet (1941a)
	Ox	—	—	8.0	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Ox	100	6.5	15.4	P estimations.	Schmidt and Thannhauser (1945)
	Rat	147–234	37–48	4.0	From pentose and desoxypentose estimations.	Schneider and Klug (1946)
	Rat	188±7.7	49.1±1.6	3.8	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
	Rabbit	108–130	44–61	2.3	Method of Schmidt and Thannhauser.	Davidson (1947)
	Cat	130–165	38–49	3.4	Method of Schmidt and Thannhauser.	Davidson (1947)
	Ox	170–185	21–22	8.1	Method of Schmidt and Thannhauser.	Davidson (1947)
	Man (one sample)	42	31	3.6	Method of Schmidt and Thannhauser.	Davidson (1947)
Kidney	Rabbit	25.5	—	—	Calculated from pentose estimations.	Brachet (1941a)
	Sheep	—	—	1.8	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Rat	47	33.5	1.4	P estimations.	Schmidt and Thannhauser (1945)
	Rat	25–30	33–43	0.7	Calculated from pentose and desoxypentose estimations.	Schneider & Klug (1946)
	Rat	42.3±0.4	40.4±1.1	1.0	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
	Rat	60	119	0.5	Calculated from pentose and desoxypentose estimated on La salts.	Euler and Hahn (1946)
Brain	Sheep	—	—	2.1	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Rat	15–20	12–13	1.5	Calculated from pentose and desoxypentose estimations.	Schneider (1945)
	Rat	33	15	2.2	P estimations.	Schmidt and Thannhauser (1945)
	Rat	20.5±0.1	19.1±1.4	1.0	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
Spleen	Ox	49	96	0.5	Calculated from pentose estimations.	Jorpes (1928a)
	Rabbit	38	—	—	Calculated from pentose estimations.	Brachet (1941a)
	Sheep	—	—	0.5	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Rat	70	54.5	1.3	P estimations.	Schmidt and Thannhauser (1945)
	Rat	37–54	115–135	0.3	From pentose and desoxypentose estimations.	Schneider and Klug (1946)
	Rat	58.4±5.7	144±15.8	0.4	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
	Rat	56	80.5	0.7	Calculated from pentose and desoxypentose estimated on La salts.	Euler and Hahn (1946)
	Rat	37	110	0.3	Calculated.	Hammarsten and Hevesy (1946)
	Rat (200–240 g.)	63–86	76–85	0.9	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rat (60–80 g.)	70–82	68–78	1.0	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rabbit	67–79	81–96	0.8	Method of Schmidt and Thannhauser.	Davidson (1947)
	Cat	84–151	73–94	1.4	Method of Schmidt and Thannhauser.	Davidson (1947)
	Man (one sample)	36	77	0.5	Method of Schmidt and Thannhauser.	Davidson (1947)
Thymus	Calf	63	378	0.2	Calculated from pentose estimations.	Jorpes (1928a)
	Rabbit	32	—	—	Calculated from pentose estimations.	Brachet (1941a)
	Sheep	—	—	0.2	Pentose and desoxypentose estimations on La salts.	Davidson and Waymouth (1944a)
	Calf	37	145	0.25	P estimations	Schmidt and Thannhauser (1945)
	Rat	29–47	240–309	0.15	From pentose and desoxypentose estimations.	Schneider and Klug (1946)
	Rat	53.3±1.5	274±10.4	0.2	Modified method of Schmidt and Thannhauser.	Schneider (1946b)
	Rat (200–240 g.)	87–116	181–242	0.5	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rat (60–80 g.)	114–135	181–261	0.6	Method of Schmidt and Thannhauser.	Davidson (1947)
	Rabbit	89–99	181–250	0.4	Method of Schmidt and Thannhauser.	Davidson (1947)
	Calf	80–100	224–250	0.4	Method of Schmidt and Thannhauser.	Davidson (1947)

ing the extracted tissue powder with hot trichloroacetic acid so as to hydrolyze the nucleic acids and split them off from the residual denatured protein. Pentose and desoxypentose estimations were then carried out on the extract.

A new approach to the problem was made by Schmidt and Thannhauser (1945), who treated the extracted tissue powder with warm alkali so as to break down the ribonucleic acid into its constituent acid soluble nucleotides without appreciably affecting the desoxyribonucleic acid. When the alkaline digest is acidified, the ribonucleotides remain in the acid-soluble fraction while the desoxyribonucleic acid is centrifuged down in the protein precipitate. The nucleic acid content of the tissue can therefore be determined from phosphorus estimations on the supernatant fluid and on the precipitate or on the original digest. Schneider (1946b) has compared this technique with a modification of his own earlier method involving pentose and desoxypentose estimations, and has found reasonably good agreement.

Recently Hammarsten and Hevesy (1946) have quoted figures for the nucleic acid content of a number of tissues without, however, giving details of their analytical procedure.

The results obtained by these different authors are summarized in Table 1.

The figures for liver tissue given by different authors show quite good agreement, the desoxyribonucleic acid phosphorus (DNAP) varying from about 20 to 30 mg. per 100 g. fresh tissue, and the ribonucleic acid phosphorus (RNAP) over a wider range from about 55 to 110 mg. per 100 g. so that the ratio of ribo- to desoxyribo- nucleic acid is in most cases of the high order of 4:1. The figures of Hammarsten and Hevesy (1946) show a lower RNAP, a higher DNAP and consequently a lower ratio than is generally found and are therefore not in accord with those of most other investigators. It should be emphasized that the nucleic acid content of any tissue, including liver, may show variations with age, sex, species, and nutritional condition, and these possible sources of discrepancies between the results of different authors must be constantly kept in mind.

In the case of pancreas, although different authors using material from a number of species have produced figures for the RNAP:DNAP ratio which cover a wide range, it is clear from all the results that this tissue contains much more ribonucleic acid than desoxyribonucleic acid.

Both liver and pancreas are tissues in which the process of protein synthesis takes place for the provision of plasma proteins or digestive enzymes respectively. The importance of ribonucleic acid in this process has been emphasized by the work of Caspersson and his colleagues (Caspersson and Santesson, 1942). In tissues such as kidney or brain, where protein synthesis does not occur to any appreciable extent, ribonucleic acid is much less abundant. The available figures for kidney tissue (Table 1) cover a wide range but most authors quote values for the RNAP:DNAP ratio of 1.0 or slightly higher. A similar wide variation is found by different authors for brain tissue and the RNAP:DNAP ratio may lie between 1.0 and 2.2.

In spleen and thymus nuclear material is abundant and desoxyribonucleic acid predominates, most authors obtaining a figure for spleen of the order of 0.5 for the ratio of RNAP:DNAP. Schmidt and Thannhauser (1945) however, quote the much higher figure of 1.3 due mainly to a lower DNAP concentration than is generally found. In a large series of investigations on rats of different ages we have consistently found figures lying between 0.6 and 1.0 for the RNAP:DNAP ratio.

A figure of about 0.2 for the ratio of RNAP: DNAP in thymus tissue is commonly found, but our own figures obtained by the method of Schmidt and Thannhauser have usually been rather higher, and values for the RNAP of more than 100 mg. per 100 g. have been consistently found with RNAP:DNAP ratios of about 0.5-0.6.

LIVER TISSUE

The liver forms one of the most useful tissues for nucleic acid investigations. It can be obtained in convenient quantities even from small laboratory animals, it is rich in nucleic acid, and it is susceptible to easily induced physiological and pathological changes. As we have already seen, liver tissue contains about four times as much ribonucleic acid as desoxyribonucleic acid, the latter being located in the nuclei and the ribonucleic acid, apart from small amounts in the nucleoli, being found mainly in the cytoplasm. The evidence for this distribution is derived from several sources.

(1) In the first place, nuclei and cytoplasm may be separated mechanically and the nucleic acids isolated from each fraction. If a tissue pulp is ground gently in saline solution so as to rupture the cell walls, cytoplasmic material and nuclei may be separated by a simple process of differential centrifugation (Claude, 1946). Ribonucleic acid may then be extracted from the cytoplasmic fractions and desoxyribonucleic acid from the nuclei. Alternatively the finely minced tissue may be thoroughly washed with 0.14 M NaCl to remove cytoplasmic nucleoprotein from which ribonucleic acid may subsequently be prepared; the residual nuclear material is then treated with M NaCl to extract the desoxyribonucleoprotein (Mirsky and Pollister, 1946). Desoxyribonucleic acid may also be prepared from nuclei isolated by the citric acid method.

Estimations by Schneider (1946c) on fractions separated by centrifugation from homogenates of liver tissue have revealed the presence of desoxyribonucleic acid only in the nuclear fraction, while ribonucleic acid was found in small amounts in this fraction, in larger amounts in the mitochondria or

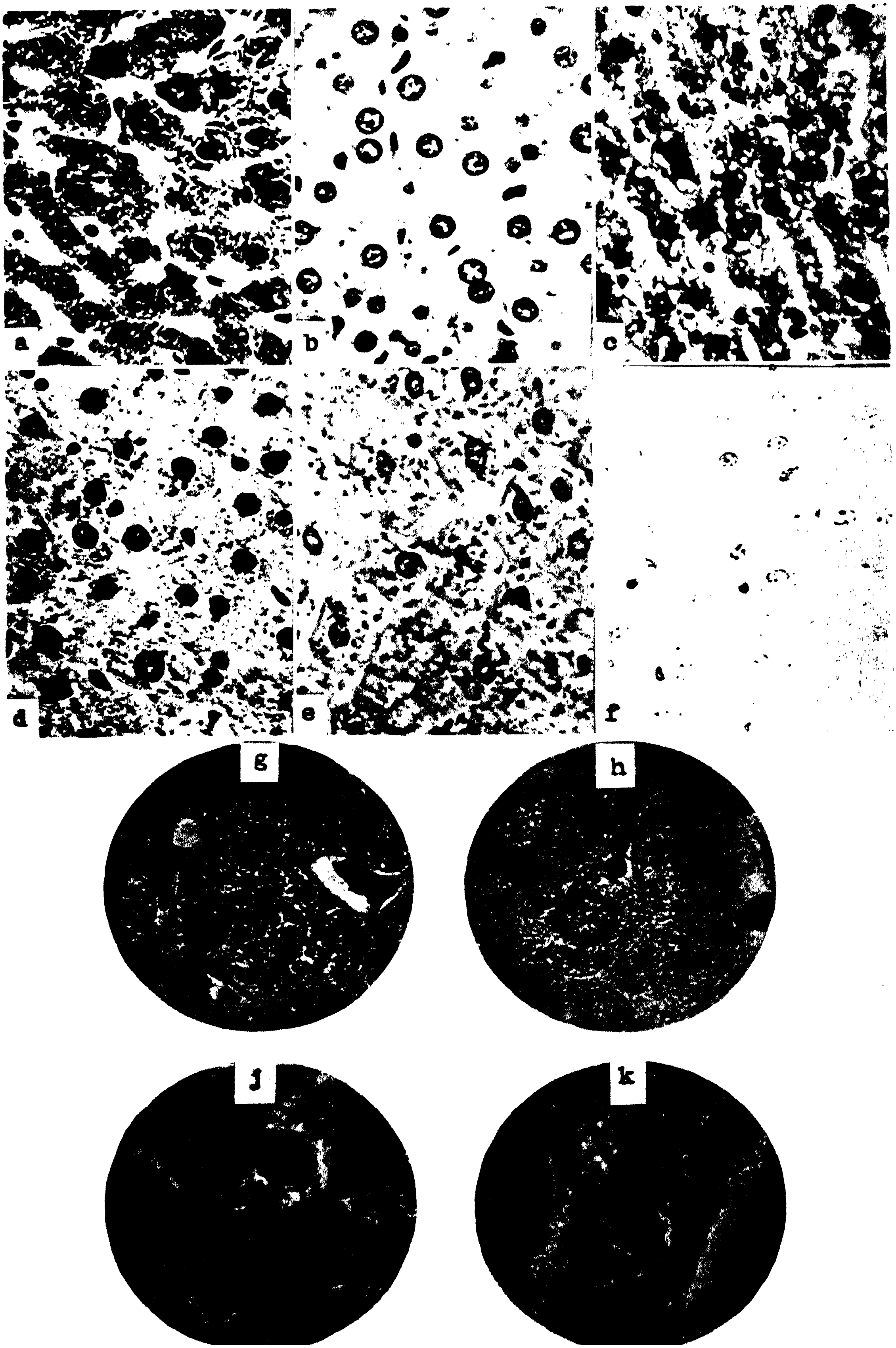
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Fig. 1.

a-f. Sections of rat liver tissue stained pyronine celestine blue, magnification × 430.

a. Section fixed in formalin and incubated in veronal acetate buffer, pH 6.75, for 3 hours at 37°. The cytoplasmic granules stain deeply with pyronine.

b. Similar section incubated under the same conditions in buffer containing 10 mg. crystalline ribonuclease per 100 ml. The cytoplasmic granules no longer stain with pyronine.

c. Section of liver tissue from a rat fasted for 48 hours, showing shrinkage of cytoplasm and loss of granular material.

d. Section of liver tissue fixed in chilled acetone and incubated in veronal acetate buffer, pH 7.25 for one hour at 37°. The cytoplasmic granules are less prominent than in *a*.

e. Similar section incubated under the same conditions in buffer solution containing 10 mg. purified desoxyribonuclease per 100 ml. The nuclei stain less intensely than in *d* but the cytoplasmic granules are still prominent.

f. Section of liver tissue fixed in chilled acetone and incubated for one hour in buffer containing ribonuclease and for one hour in buffer containing desoxyribonuclease.

g-k. Sections of liver tissue from a well fed rat, unstained, photographed in ultraviolet light at 257 mμ × 1100

g. Section of liver tissue, fixed in formalin and cut at about 1μ, incubated for five hours at 37° in isotonic veronal acetate buffer pH 6.75. The nucleoli and the cytoplasmic granules absorb ultraviolet light intensely.

h. Similar section incubated under the same conditions in buffer containing 100 mg. crystalline ribonuclease per 100 ml. The cytoplasmic masses no longer absorb ultraviolet light although their outlines can be faintly distinguished.

j. Section of liver tissue, fixed in chilled acetone and cut at 3μ, incubated for two hours at 37° in isotonic veronal acetate buffer pH 6.0. The nuclei absorb ultraviolet light intensely, especially in the region of the nuclear membrane.

k. Similar section incubated under the same conditions in buffer containing 10 mg. purified desoxyribonuclease per 100 ml. The nuclei no longer absorb ultraviolet iglht so intensely as in *j*. The cytoplasmic masses still show absorption. (The ultraviolet photographs were made by Mr. F. V. Welch of the National Institute for Medical Research, London. *g* and *h* are reproduced, by permission, from the *Journal of Physiology*, 1946, *105:* 191).

large granule fraction, and most abundantly in the unfractionated cytoplasmic residue.

(2) The ribonuclease histochemical test, first described by Brachet (1940, 1941b), forms a useful means of confirming the presence of ribonucleic acid in the cytoplasmic particles of the liver cell (Davidson and Waymouth, 1944d), so long as it is realized that the use of enzymes in histochemical tests is beset with pitfalls and that the results should be interpreted with great caution. The results of a ribonuclease test on sections of liver tissue from a well fed rat are illustrated in Fig. 1. After staining with pyronine and celestine blue as recommended by Sanders (1946) the basophilic cytoplasmic particles stain deeply with pyronine in the control section, incubated in buffer alone (Fig. 1a), whereas in the section incubated in the same buffer with the addition of crystalline ribonuclease the nuclei stain as before with celestine blue although the cytoplasm no longer takes up the pyronine (Fig. 1b). Ribonucleic acid would therefore appear to be located in the cytoplasmic particles. The ribonuclease test obviously depends on the specificity of the enzyme for ribonucleic acid and in this connection it should be emphasized that liver ribonucleic acid is known to act as a substrate for ribonuclease (Davidson and Waymouth, 1944b) and that the enzyme is able to split off ribonucleic acid from isolated cytoplasmic particles (Schneider, 1946a).

Purified desoxyribonuclease prepared from pancreas by the method of McCarty (1946), has been used by Sanders (1946) and Holmes (1946) to remove desoxyribonucleic acid from cell nuclei. For liver we have found this test much less satisfactory than the ribonuclease test since desoxyribonuclease does not appear to effect complete and selective removal of desoxyribonucleic acid from sections of liver tissue and the results vary widely according to the fixative employed. After fixation with drastic agents such as Susa, or even with formalin, desoxyribonuclease in low concentrations exerts very little action, but if chilled acetone is used as fixative, desoxyribonuclease is able to remove some of the stainable material from the nuclei (Fig. 1d and e). The nuclear membrane and the nucleolus still stain faintly after enzyme treatment, although the cytoplasm is much the same in control and enzyme treated sections. With acetone fixation however, some cytoplasmic material is readily lost even from control sections incubated in buffer alone. Stowell (1946) records the partial removal of Feulgen positive material from the nuclei in sections incubated with desoxyribonuclease.

Treatment with both ribonuclease and desoxyribonuclease removes nearly all basophilic material from liver sections which then show only very faint staining with pyronine and celestine blue (Fig. 1f).

(3) In the third place it is possible to take advantage of the high ultraviolet absorption of the nucleic acids. Ultraviolet photographs of liver cells taken in the apparatus of Barnard and Welch (1936) at the National Institute for Medical Research, London, using ultraviolet light of wavelength 257 mμ are shown in Fig. 1g-k. They reveal the presence in the cytoplasm of masses of material which absorbs ultraviolet light strongly (Davidson and Waymouth, 1946b). These masses are about 2μ in diameter (Fig. 1g) and therefore correspond in size to the "large particles" isolated by Claude (1943a and b, 1946) from cytoplasmic material. When a section of liver tissue is treated with ribonuclease before being photographed in the ultraviolet microscope (Fig. 1h), the cytoplasmic particles no longer absorb ultraviolet light although the areas which they have occupied can still be made out. On the other hand incubation of a section of liver tissue (fixed in acetone) with desoxyribonuclease results in a considerable reduction in the amount of absorbing material in the nucleus (Fig. 1j and k).

A further point which has emerged from these investigations relates to the composition of the nucleolus. Histochemical tests (Brachet, 1941b) have already shown that the basophilia of the nucleolus of the liver cell is reduced by ribonuclease treatment, and the presence of ribonucleic acid is confirmed by ultraviolet photography. In the control section shown in Fig. 1g the nucleolus absorbs the ultraviolet light intensely and uniformly over its surface, whereas in the section treated with ribonuclease (Fig. 1h) the central part of the nucleolus absorbs ultraviolet light less intensely than does the peripheral region. This suggests that any ribonucleic acid present in the nucleolus of the rat liver cell is located, in general, in the central portions, whereas the peripheral regions probably consist essentially of desoxyribonucleic acid. This conclusion is supported by the fact that the Feulgen reaction is more intense at the periphery of the nucleolus than at the centre (Davidson and Waymouth, 1946b; Stowell, 1946) and that the central part of the nucleolus stains less deeply in ribonuclease treated sections (Fig. 1b), although it still retains its basophilia in desoxyribonuclease treated sections (Fig. 1e).

It has been known for a long time that alteration in diet exerts a profound influence on the histological picture of the liver. Berg (1914, 1926) observed granules in the cytoplasm which diminished on fasting and similar observations were made by Elman, Smith and Sachar (1943). Fig. 2 illustrates the loss of granules and the shrinkage of cytoplasm in the liver cell on fasting. If, as appears probable, these granules are phospholipin-ribonucleoprotein particles we should expect that fasting would be accompanied by a drop in liver ribonucleic acid (Davidson and Waymouth, 1944c, Davidson, 1945). Fig. 2 illustrates the results of an experiment of this type in which three groups of rats were employed,

the first being used as controls, while the second group was fasted for two days and the third group was fasted for two days and then fed for a further two days. The body weight fell during fasting and was not quite restored by two days' feeding. On

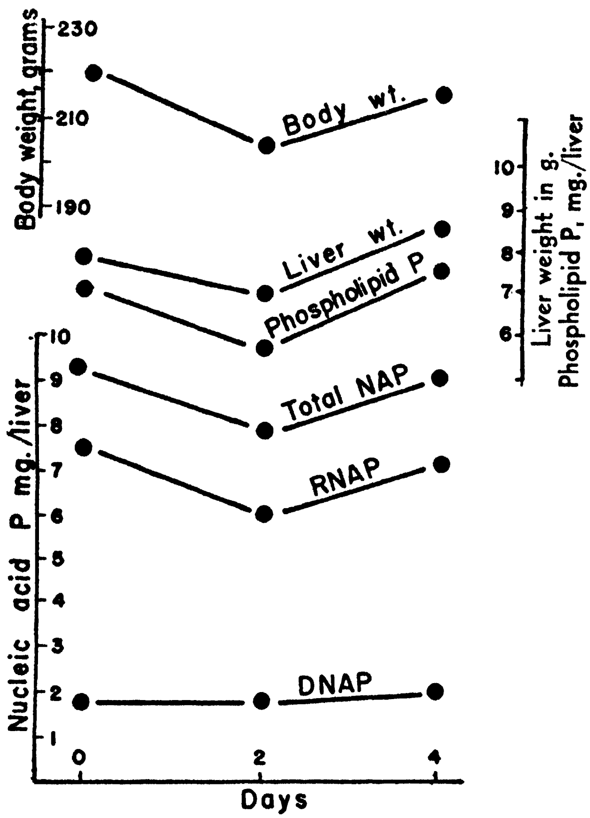

FIG. 2. The effect of two days starvation followed by two days feeding on the livers of groups of young male rats.

the other hand the weight of the liver and its phospholipin content fell during fasting and rose on feeding to a level slightly above the initial value. The total nucleic acid content of the liver also fell on fasting and rose on feeding, and these changes were due entirely to a fall and rise in ribonucleic acid, the desoxyribonucleic acid content remaining unchanged. Similar observations have been made by Brachet, Jeener, Rosseelt and Thonet (1946) who observed a fall in the pentose content of the mouse liver after fasting.

The loss from the liver cell on fasting of protein (Addis, Poo and Lew, 1936), phospholipin, and ribonucleic acid, together with the disappearance of basophilic cytoplasmic granules, has justified the conclusion that some of the phospholipin-ribonucleoproteins in the cell cytoplasm form a relatively labile store of protein-containing material. This has been referred to as "labile liver cytoplasm" by Kosterlitz (1944) who has also recorded that rats fed on a protein-poor, or protein-free, diet show a similar loss of basophilic cytoplasmic granules accompanied by a decrease in ribonucleic acid without any change in desoxyribonucleic acid (Campbell and Kosterlitz, 1947).

One method of confirming the apparent lability of the cytoplasmic ribonucleoproteins of the liver is obviously by the use of P^{32} and the results of Brues, Tracy and Cohn (1944) and of Hammarsten and Hevesy (1946) show that in the normal rat liver, the ribonucleic acid is metabolically much more active than the desoxyribonucleic acid.

Our own results shown in Table 2 amply confirm this. Sodium phosphate containing P^{32} was administered to rats in doses of about 10μc per animal. After two (or four) hours the livers were removed, cytoplasmic and nuclear material were separated,

TABLE 2. RATIO OF SPECIFIC ACTIVITY OF RIBONUCLEIC ACID TO SPECIFIC ACTIVITY OF DESOXYRIBONUCLEIC ACID IN THE LIVERS OF ANIMALS RECEIVING P^{32} AS INORGANIC PHOSPHATE BY SUBCUTANEOUS INJECTION

	Time after administration of P^{32} (hours)	Ratio
Normal rat liver	2	7.4, 6.9, 7.2
Regenerating rat liver (3rd day)	2	1.6, 2.7
Rabbit liver	see footnote*	5.6
Pregnant rabbit liver	4	7.3, 10.0
Fetal rabbit liver	2	0.2
	4	0.8, 1.6

* P^{32} concentration in the blood maintained at a high level for 9 days (specimen kindly supplied by Mr. G. L. Ada).

and the nucleic acids were isolated from each fraction. The phosphorus of the ribonucleic acid was found to be much more active than that in the desoxyribonucleic acid, the ratio of the specific activities being about 7:1. Even higher figures are given by Hammarsten and Hevesy (1946). In the nucleic acids from the liver of a rabbit which had received P^{32} over a period of several days the ratio was slightly lower (5.6:1) and of the same order as that found by Brues, Tracy and Cohn (1944) in somewhat similar circumstances. In confirmation of their results on regenerating liver, the ratio was found to be much lower for the nucleic acids isolated from the regenerating rat liver, owing to the greater activity of the desoxyribonucleic acid in this material. The nucleic acids from pregnant rabbit liver gave a ratio similar to that found in the liver of normal rats, but in the fetal rabbit the ratio was very low owing to the high activity of the desoxyribonucleic acid, which in this tissue was sometimes even more active than the ribonucleic acid. Similar results were found in an experiment with N^{15} (Table 3). When N^{15} in the form of ammonium citrate was

fed to rats or pigeons and the N^{15} content of the nucleic acids isolated from the liver was subsequently determined, the isotope was found in far greater abundance in the ribonucleic acid than in the desoxyribonucleic acid fraction. The experiments of Barnes and Schoenheimer (1943) with N^{15} did not involve separation of the two nucleic acids.

TABLE 3. RESULTS OF ORAL ADMINISTRATION OF AMMONIUM CITRATE CONTAINING 4.18 ATOM % EXCESS N^{15} TO PIGEONS AND RATS AT THE RATE OF 50 MG. PER DAY FOR 3 DAYS.

	Atom % excess N^{15}
Pigeon Liver	
Ribonucleic acid	0.048
Desoxyribonucleic acid	0.003
Uric acid excreted in last 24 hours	0.204
Rat Liver	
Ribonucleic acid	0.053
Desoxyribonucleic acid	negligible
Rat Liver (animals fasted 2 days prior to N^{15} administration)	
Ribonucleic acid	0.077
Desoxyribonucleic acid	negligible

FIBROBLAST CULTURES

One use to which determinations of nucleoprotein phosphorus (NPP) have been put is in the estimation of the amount of protoplasm in small samples of tissue. Berenblum, Chain and Heatley (1939) suggested that NPP determinations might give a better index of the amount of tissue used in a given experiment than, say, estimations of dry weight and such an index has been extensively employed with fibroblasts or "mechanocytes" growing in tissue culture (Davidson and Waymouth, 1943b, 1945, 1946a).

In the quantitative estimation of the growth of cells *in vitro* a number of criteria have been employed by various investigators, *e.g.*, measurements of the area of the culture, of the mitotic index, of the mass of the culture or its metabolic activity, but objections can be raised to all of these procedures. More recently Cunningham and Kirk (1942) have suggested an elaborate method involving measurements of the area of the culture, its thickness, the number of cells present and the average cell size, while Brues, Rathbun and Cohn (1944) have evolved a method dependent upon estimations of phosphorus depletion in the medium.

In 1942 Willmer suggested that measurements of nucleoprotein phosphorus (NPP) should give an estimate of the amount of nuclear material, or at least of cellular material, present in the cultures, and a similar method was suggested shortly afterwards by Cunningham and Kirk (1942). At this time the ubiquity of ribonucleic acid in animal cells was not fully appreciated nor was it realized that in many cells there might be much more ribonucleic acid in the cytoplasm than desoxyribonucleic acid in the nucleus. Since subsequent investigations (Davidson, Leslie and Waymouth, 1947) have revealed that the ribonucleic acid:desoxyribonucleic acid ratio for heart fibroblasts in culture may vary from 2.5:1 to 4:1 according to the nutritional state of the culture, the interpretation of results obtained by this method of assessing "growth" may have to be modified in the light of recent evidence.

In the method of Willmer (1942) the tissue is explanted in plasma clot in a constricted roller tube closed by a rubber bung and rotated slowly in the incubator. With each revolution of the tube the fluid phase, *e.g.*, embryo extract or other growth promoting substance, washes over the surface of the cultures. At the end of the growth period the roller tube becomes a test tube in which acid soluble phosphorus and lipid phosphorus are extracted from the culture which is finally ashed with perchloric acid. Residual or nucleoprotein phosphorus (NPP) is then determined by the method of Berenblum and Chain (1938). The whole procedure suffers from the disadvantage that the culture must be destroyed in order to estimate the amount of its growth, but this disadvantage is shared by some other methods of growth estimations and is in any case outweighed by some of the advantages of this particular method.

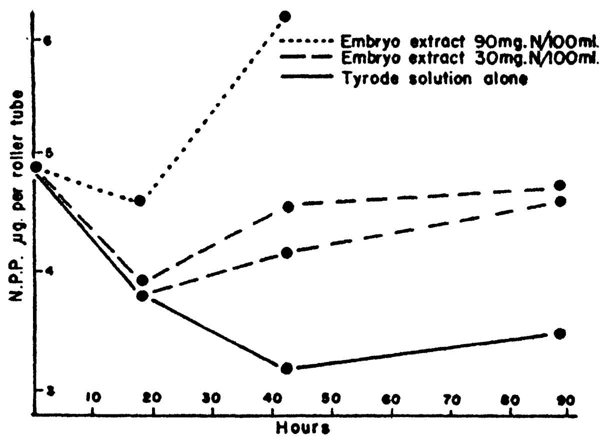

FIG. 3. The nucleoprotein phosphorus (NPP) content of tissue cultures maintained in plasma clot in roller tubes with Tyrode solution or embryo extract as fluid phase. Each roller tube contained 12 explants from 10 day old chick embryo heart.

In our own experiments with this technique (Davidson and Waymouth, 1943b, 1945, 1946a) we have restricted ourselves to the use of fresh explants from the chick embryo heart, usually of 9-10 days. When such explants are set up in plasma clot in roller tubes which contain Tyrode solution alone as a fluid phase, a steady loss of NPP occurs over a period of 48 hours or more (Fig. 3). This

loss is due in part at least to the washing out of cellular material from the damaged cells at the cut edges of the explant, and has been observed by Berenblum, Chain and Heatley (1939) in tissue slices suspended in Tyrode solution, and by Willmer (1942) in the case of heart fibroblast cultures. This initial loss is not prevented by the presence of embryo extract in the roller tubes, although it can be diminished to some extent if the concentration of embryo extract is high (Fig. 3). In the presence of embryo extract the NPP of the culture reaches a minimum in less than 24 hours after the cultures are set up and then begins to rise. If the concentration of embryo extract is high, the rise is sharp and in two days the NPP may considerably exceed the value found at the time of setting up (Fig. 3). With moderate concentrations of embryo extract the rise is less marked but is nevertheless significant and the value for the NPP tends to reach a fairly steady level after two days. When embryo extract in moderate concentration is added to cultures which have been maintained in Tyrode solution alone for 18 hours, a rise in NPP occurs very quickly and the final figure is of the same order as is found when embryo extract has been present in moderate concentration from the start (Fig. 3).

It should be noted that the NPP in such cultures will not go on rising indefinitely even with frequent renewals of embryo extract in the fluid phase. After some four days the NPP tends to remain constant or even to fall. Willmer (1942) has suggested that embryo extract accelerates the rate of synthesis of nucleoprotein but that the actual amount synthesized is limited by the supply of some other factor derived probably from the plasma. In the case of fourth passage fibroblast cultures he has found a steady drop in NPP even in the presence of 30% embryo extract.

Support for the validity of this method of estimating growth is given by the good correlation found by Willmer (1942) between NPP and dry weight in osteoblast cultures, but it should be emphasized that there appears to be little or no correlation between NPP level and the occurrence of mitosis since mitotic activity may be pronounced while NPP is actually falling. Brues, Rathbun and Cohn (1942) have observed a high peripheral growth rate in conjunction with a declining phosphorus content, and our own experiments have shown that increase in the area of the culture may coincide with a fall in NPP, although, since our cultures are made with fresh explants, area measurements are of very limited significance.

These results have emphasized the need for a more detailed study of the changes in nucleic acid content of growing fibroblasts, and for the separate estimation of the two types of nucleic acid in them. The main difficulty has been to obtain sufficient material for chemical analysis, but by pooling the tissue from a large number of cultures and by adapting the method of Schmidt and Thannhauser (1944) to minute amounts of tissue, it has been found possible to follow the changes in the ribonucleic acid and desoxyribonucleic acid separately (Davidson, Leslie and Waymouth, 1947). The chief feature which has emerged from this investigation is that, not unexpectedly, ribonucleic acid varies more readily than does desoxyribonucleic acid.

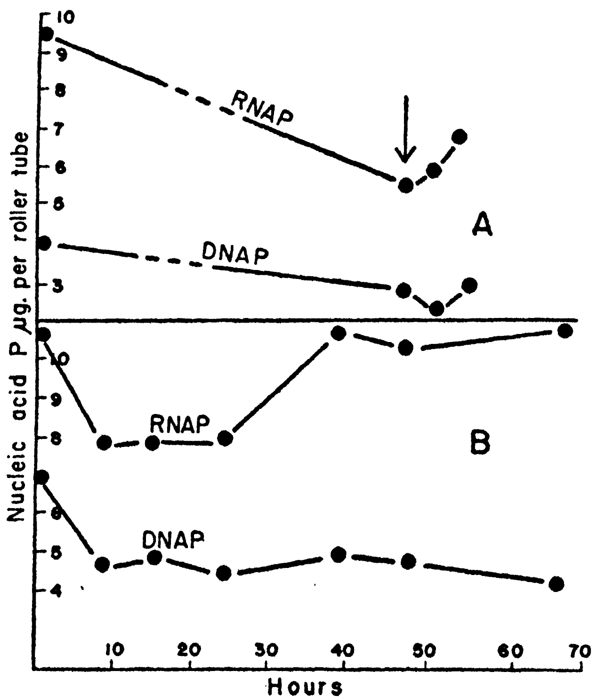

FIG. 4. Ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) in tissue cultures maintained in plasma clot in roller tubes. Each roller tube contained 24 explants from the twelve day old chick embryo heart. In A the fluid phase for the first 48 hours consisted of Tyrode solution alone. Embryo extract and serum were added at the time indicated by the arrow. In B embryo extract was present from the time of setting up.

When embryo extract is added to cultures which have been maintained for 24-48 hours in Tyrode solution alone, a sharp rise in total nucleoprotein phosphorus occurs and this rise is due to an increase in RNAP (Fig. 4). Changes in DNAP are very small in comparison with changes in RNAP so that the RNAP:DNAP ratio rises. It is not uncommon to find regular fluctuations in the RNAP level during the general rise which follows the addition of growth promoting media. Although very significant changes in the nucleic acid level of the cultures occur in the first few hours after the application of such media, alterations in the naked-eye (or microscopic) appearance of the cultures are not prominent in the early stages and are most pronounced during the second 12 hours of this period.

The Nucleic Acid Content of Bone Marrow

The bone marrow forms a labile tissue readily susceptible to physiological and pathological changes involving alteration in the nucleic acid content of the different types of cells which it contains. Although the changes in histological appearance can readily be followed by microscopical examination, it is not easy to follow the corresponding chemical changes owing to the difficulties of obtaining from the living human subject samples of sufficient size for accurate and analytical work. Such samples, moreover, tend to be mixed with varying amounts of fat and blood. By adopting methods similar to those employed for fibroblasts it has, however, been found possible to estimate the nucleic acid content of marrow tissue and to correlate it with the histological appearances (Davidson, Leslie and White, 1947b).

In a recent series of investigations 0.1-0.2 ml. of marrow obtained from human subjects by sternal puncture has been used for histological examination and for chemical analysis. Sections were stained with pyronine-methyl green and by other methods before and after ribonuclease treatment, while chemical analysis was carried out by a modification of the technique of Schmidt and Thannhauser (1945). The results of such examinations in normal subjects have shown that primitive free cells are few in number, partly differentiated forms (erythroblasts and myelocytes) are more numerous, while normoblasts and band-form granulocytes are still more abundant. The cytoplasmic basophilia removable by ribonuclease diminishes very rapidly with the maturation of the older erythroblasts and granulocytic cells (White, 1947).

The results for the nucleic acid estimations on sternal marrow from ten normal individuals have shown that the mean value for the total nucleic acid phosphorus (NPP) is 20.7 mg. per 100 g. fresh tissue (range 13.0-29.0); for RNAP the mean is 14.2 (range 10.3-18.3) and for DNAP it is 6.9 (range 2.7-13.0) (Davidson, Leslie and White, 1947a). Although the values for normal subjects vary over a wide range this is scarcely surprising in a rather heterogeneous tissue contaminated with more or less blood and fat. In pathological conditions figures far outside this range are commonly found.

In pernicious anemia the marrow shows evidence of hyperplastic and megaloblastic changes with prominent basophilic haemocytoblasts, proerythroblasts and megaloblasts. The figures for both RNAP and DNAP are high and may exceed three times the standard division of the normal. As the result of the application of specific therapy (liver extract or folic acid) striking changes occur. At the height of the reticulocyte response the histological picture is altered so that a predominance of erythroblasts and normoblasts is found, with a reduction in haemocytoblasts and megaloblasts. The change is thus to a less basophilic type of cell but the cellularity of the tissue, as seen in sections is unaltered. Nevertheless the nucleic acid concentration shows a marked fall, chiefly in RNAP, corresponding to the change in the stage and maturation of the cells. Later on, the cellularity of the marrow reverts to normal and the nucleic acid levels fall to within the normal range (Fig. 5).

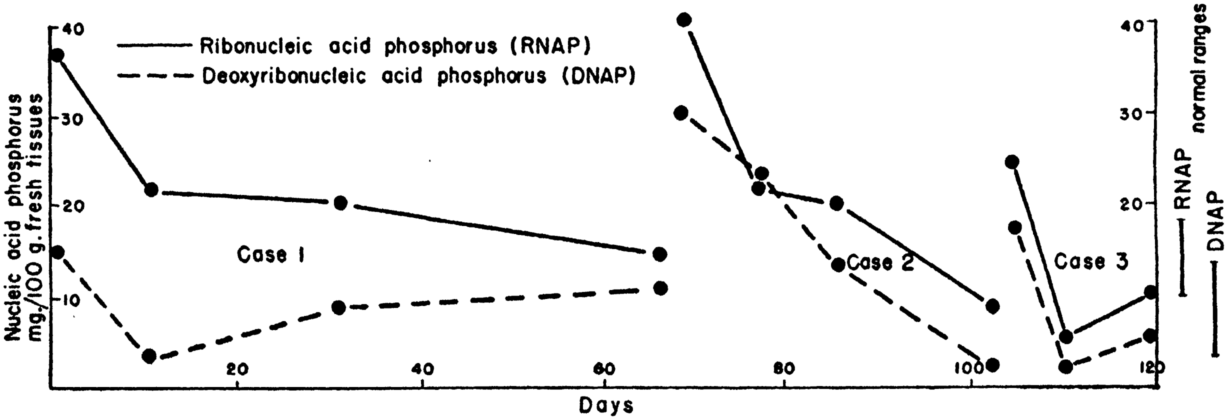

Fig. 5. The effect of specific therapy (liver extract and folic acid) on the nucleic acid concentration in the sternal marrow in three cases of pernicious anemia.

In other pathological conditions striking changes may also occur in which the correlation between the histological picture and the nucleic acid content is well marked. A case of haemolytic anemia with hyperplastic marrow showing erythropoietic activity with predominance of the earlier cell types showed a total NPP of 71.4 mg. per 100 g. fresh tissue with an RNAP of 56.0 and a DNAP of 15.4, while one case of lymphatic leukemia gave a total NPP concentration of 216 mg, per 100 g. with an RNAP of 95 and a DNAP of 121. Another example of an instance in which the RNAP/DNAP ratio was less than 1 was found in a patient with idiopathic micro-

cytic hypochromic anemia, with a slightly hyperplastic marrow containing many poorly haemoglobinized intermediate erythroblasts.

The chief pattern which has emerged from these investigations is that ribonucleic acid is rich in the cytoplasm and nucleoli of the youngest free haemopoietic cells and the amount diminishes progressively with cellular development. In general, ribonucleic acid is more abundant in the red cell series than in the granulocytic series and is greatest in the proerythroblast which has an extensively basophilic cytoplasm comparable only with that of the plasma cell. Ribonucleic acid disappears with the appearance of haemoglobin or specific granules in the cell and as it disappears from the nucleolus the nucleolar associated chromatin makes its appearance (Thorell, 1944; White, 1947). Evidence obtained here indicates that ribonucleic acid is abundant in those cells which are engaged in synthesizing protein, either haemoglobin or the protein of the specific granules, and this is in accordance with the well known views of Caspersson and his colleagues (*c.f.* Caspersson and Santesson, 1942) on the role of the cytoplasmic and nucleolar ribonucleoproteins in the process of protein synthesis.

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DISCUSSION

STOWELL: Several years ago I examined the blood cells of a few patients with lymphoid leukemia and found that, as determined photometrically by the absorption of complementary light by the Feulgen reaction, the individual leukemic cells contained significantly increased amounts of desoxyribonucleic acid as compared with normal lymphocytes.

While working in Caspersson's laboratory in Stockholm, I carried out several experiments on the rat liver to observe the cytochemical and morphologic variations in nucleoli and nucleic acid under different conditions of growth and protein formation. Some sections of fixed and frozen-dried livers were stained and others used for microspectrophotometry and photographed with ultraviolet light of 257 or 275 millimicrons wave length.

In rats on a protein free diet for three months, the nuclei and cytoplasmic volume of hepatic cells were decreased while the nucleoli doubled in size. When placed on a high protein diet the number of nucleoli per nuclear section increased and their mean size decreased.

That the observed increased absorption of ultraviolet light of 257 millimicrons in the cytoplasm indicated larger amounts of ribonucleic acid in regenerating cells was shown by macrochemical analyses on the same material by Professor Hammarsten. A study at 6-hour intervals during the first 48 hours of regeneration showed that the volume of the nucleoli could increase by three and a half or more times at 24 hours. The nuclear volume was also increased in the regenerating liver. Fasting for 24 hours caused a decrease to one half in nucleolar volume. Hepatomas were produced in other rats fed p-dimethylaminoazobenzene and great variations in the nucleoli and nucleic acids were observed in these regenerating and neoplastic cells.

These variations in nucleoli and ribonucleic acids under conditions of low and high protein diet, in rapidly proliferating regenerating liver and in aberrantly growing neoplastic tissue are of interest as further confirmatory evidence of the relationship of nucleoli and nucleic acid in protein formation.

DAVIDSON: We have not studied the effects of nutritional changes upon the liver over such long periods as Dr. Stowell, but I understand that Dr. Brachet has made some investigations on this subject.

BRACHET: We only studied the effect of fasting on the ribonucleoprotein content, and did not look for possible changes when feeding was resumed; our results on the pyronine stainability and the pentose content of the liver during fasting agree perfectly well with yours.

IN VITRO AND IN SITU ACTION OF IONIZING RADIATIONS ON NUCLEOPROTEINS OF THE CELL NUCLEUS

MAURICE ERRERA[1]

The reason for studying the action of ionizing radiations on chromosome constituents is to find out something of the fundamental phenomena occurring during the production of biological and morphological anomalies by radiations (*i.e.*, lethal effects, mutations and breakages or translocation of chromosomes). These biological and morphological effects have been extensively studied and a relationship between dosage and the effects produced has been observed. The interpretation of these findings has led certain authors to postulate a direct effect of these radiations; thus a single ionization would suffice to cause a mutation, a single ionizing particle would be capable of breaking a chromosome. The criteria of such direct action of radiations have been clearly expressed by Lea (1946). It is therefore of interest to observe some molecular components, which, when submitted to these radiations in a state similar to that in which they exist in the living cell, will serve as a model for conditions presumed to exist in the latter.

It has unfortunately been impossible so far to study quantitatively these chromosomal components in the organized state in which they exist in living cells, by direct micromanipulation or other methods.

Nevertheless, we have attempted to approach this problem by an indirect method which will be briefly discussed.

I. Material and Methods

If isolated nuclei[2] from chicken erythrocytes are dispersed in an appropriate buffer solution[3] the nuclei swell into a homogeneous gel, the rigidity of which can be determined by appropriate methods (Jeener, 1946b).

We used the simple method (Balasse-Jeener) which consists of comparing the period of oscillation in air (T_0) of a small torsion pendulum, to T_1, the period with which the same pendulum oscillates when dipping into the solution to be studied. Results can be expressed in arbitrary units given by the equation $E = \frac{T_0}{T_1} - 1$; and E was found to vary linearly with the concentration which we determined on the basis of thymonucleic acid, by the Dische-Davidson method (Davidson and Waymouth, 1944). Jeener (1946a) showed this gel formation to be intimately related to the presence of thymonucleic acid, since the rigidity disappeared rapidly (in a few minutes) in the presence of desoxyribonuclease activated by MG^{++}. This phenomenon is accompanied by loss of flow birefringence. In the presence of crude trypsin, the loss of rigidity occurs, but much more slowly, and the resulting solution retains its negative birefringence. These results suggest that thymonucleic acid as well as protein is involved in the formation of the rigid gels. Fractionation by ultracentrifugation of this material shows that the proteins are at least of two kinds: (1) the histone which stays in the supernatant with the thymonucleic acid and (2) a protein containing tryptophane which sediments and to which is bound the alkaline phosphatase of the nucleus (Jeener, 1947). As impurities the material contains all the dialyzable substances of the nuclei, but these do not affect the experiments to be described, the results obtained being identical on dialyzed and on fresh gels.

II. In Vitro Irradiation of Nucleic Acid Complexes

When submitted to the action of hard X-rays, the rigidity of the nucleoprotein gels described above completely disappears if sufficient dosage is used. The following curves describe the results so obtained for gels of different concentrations (Fig. 1). The ordinates are given as the per cent of the first reading ($\frac{E}{E_0} \times 100 = \varepsilon$) plotted on a logarithmic scale. The abscissae represent times of irradiation (the dosage being 1000 r per minute). Each experimental point is the arithmetic mean of five experi-

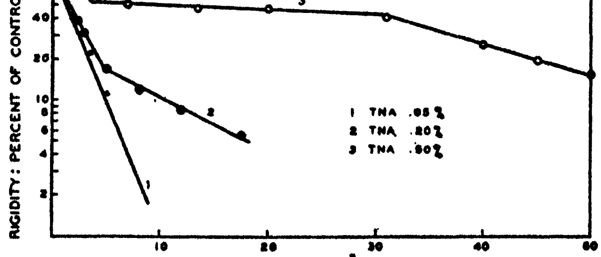

Fig. 1.

[1] This work was done with a fellowship from the Fonds National de la Recherche Scientifique, in the laboratories of Professors J. Brachet and P. Gerard, University of Brussels, Belgium.

[2] By freezing and thawing.

[3] We generally used 0.6 M NaCl buffered to pH 8.6 by a dilute carbonate bicarbonate system; but one can use pure water, the pH of which has been raised to 10.5 by NaOH.

ments done on the same batch of nuclei. For dilute solutions (0.05 gr. TNA%) the decrease in rigidity follows an exponential relation. If, however, the concentration of thymonucleic acid is raised to 2% the curve becomes more complicated. After 5000 r the decrease which was almost exponential is slowed down. This is further emphasized when the concentration of the solution is 0.5%.

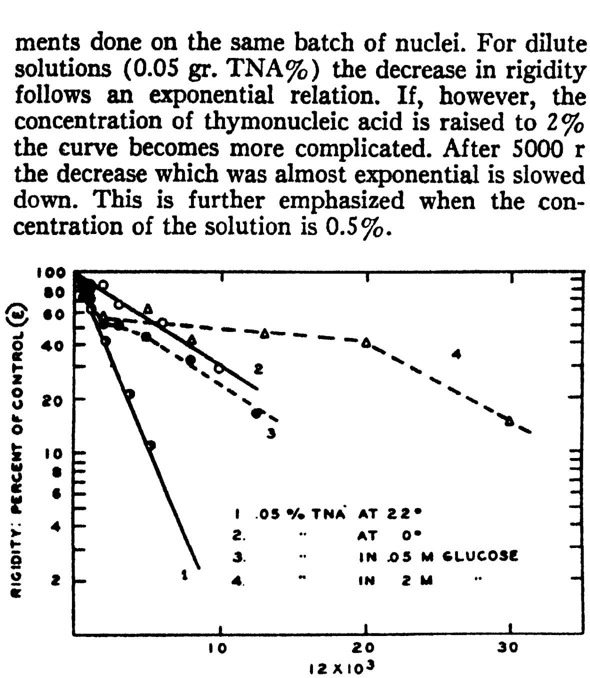

FIG. 2.

Interpreted on the basis of Dale's (1943) results, the action of ionizing radiations on dilute solutions of macromolecular compounds is chiefly due to an indirect effect produced through some kind of activation of water molecules (Weiss, 1944). If this be true, the protecting effect of different compounds competing for activated water molecules should also be found in the present instance. As protecting agent we used glucose and the results of such experiments are shown in Fig. 2 (curves 3 and 4): a definite protecting effect is found. To reduce the rigidity to 37% of its original value 20,000 r are needed in a solution containing 2% glucose instead of 2000 r in the control experiment run in absence of glucose (curve 1). These results suggest that the irregular curves obtained for solutions containing 0.2 and 0.5% thymonucleic acid may be due to some protecting agent occurring in the form of a breakdown product of irradiation.

Another consequence which should be expected for an indirect effect of radiation on the complex studied would be the existence of a temperature coefficient greater than 1.

In Fig. 2 we have also included data for a run made at 4° C (irradiation and subsequent measurements were made at that temperature). The temperature coefficient for a difference of 18° C (Q 18) calculated from these results is approximately 1.6. The same value has been found for a 0.2% thymonucleic acid gel; while a value of 1.3 was found for a 0.5% thymonucleic acid concentration.

After irradiation we noted a decay phenomenon in these solutions, for the rigidity continues to drop on standing. This is a phenomenon strikingly similar to the one described by Taylor, Greenstein, and Hollaender in this volume.

Fig. 3 shows experimental data for nucleoprotein gels containing respectively 0.3% and 0.17% thymonucleic acid after 20,000 r in the first instance and 10,000 r in the second. (Points represent here a single experiment.)

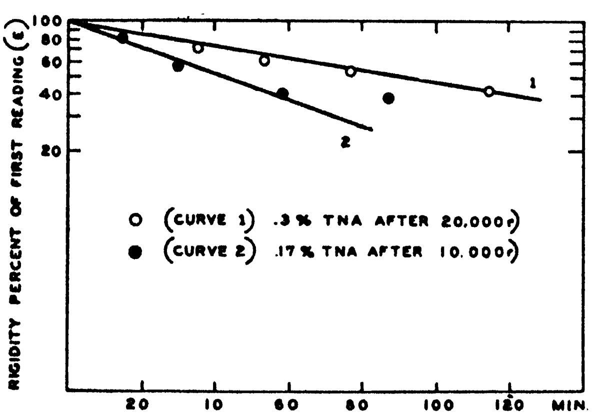

FIG. 3.

III. IN SITU EXPERIMENTS

The results reported in the above permit only a limited biological interpretation since they are obtained on *in vitro* irradiation of nucleoprotein complexes of the cell nuclei. However, the nucleoprotein gels can be obtained from cells which have been irradiated prior to any other treatment and then studied.

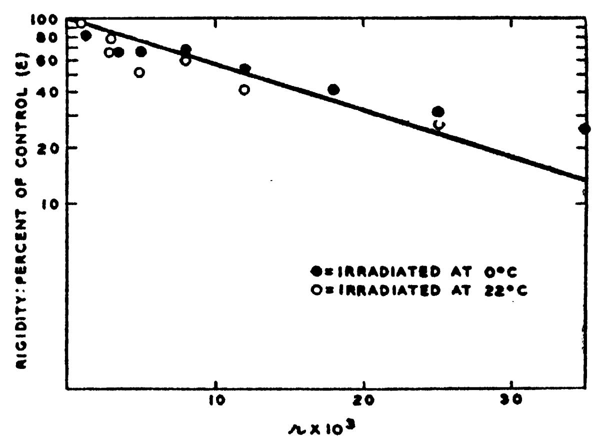

FIG. 4.

Aliquots of washed nucleated erythrocytes were submitted to different doses of radiation (1000 r per minute); these were then frozen for 10 minutes

in dry ice, thawed at 30° and washed several times by centrifugation in physiological saline, until the supernatant was free from hemoglobin. An identical volume of each batch was then dispersed in 0.6 M KCl at pH 8.6 and kept in the ice box for 24-48 hours before rigidity determinations were made. A determination of thymonucleic acid was made on each batch and values of E were corrected for variation in concentration. It was found (Fig. 4) that the resulting gels were proportionally less rigid when the corresponding cells had been irradiated longer.

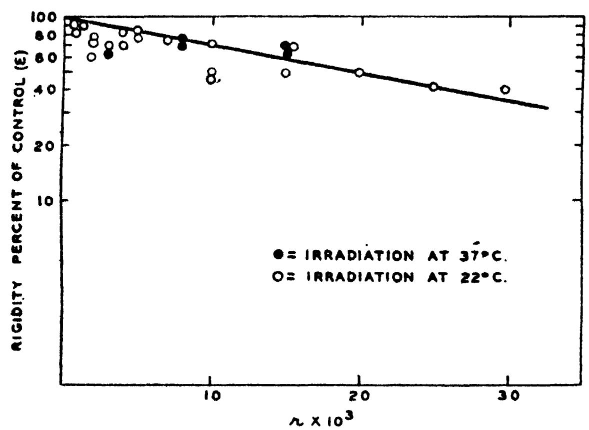

Fig. 5.

Similar results were obtained when isolated nuclei were irradiated prior to their dispersion into the salt solution (Fig. 5).

The experimental data are not yet sufficient and the method is not yet accurate enough to permit us a clear analysis of the curve obtained, although the points are roughly placed on a straight line in semi-logarithmic plotting, which would mean an exponential-like curve. A striking fact is that for a dosage of about 5000 r about 20% of the rigidity is lost; unfortunately not enough is known yet about molecular constituents of the gels studied to be able to interpret these results in any way, as far as structural changes in the molecular components are concerned. But the least one can conclude is that for these dosages considerable alteration is accomplished in the thymonucleic acid complex studied.

Temperature coefficient: The experimental points are not displaced to any appreciable extent if irradiation is performed at 4° or 37° C. This implies that the temperature coefficient for the action of radiation on these properties of the nucleoproteins is approximately equal to 1. These two facts, namely the exponential-like curve and temperature coefficient of 1, suggest a phenomenon of a fundamentally physical nature, that is, a direct action of radiation on the susceptible components of the cell.

Discussion

It has been shown that nucleoprotein complexes of the nucleus are strongly affected when irradiated in situ. We have noted that when irradiated in very dilute solution the nucleoprotein complex is altered with a temperature coefficient greater than 1 ($Q18 = 1.6$). If the concentration is raised, this coefficient tends to become lower ($Q18 = 1.3$ for 0.5% solutions). Finally when irradiated in situ this coefficient can be stated to be close to 1.

Table 1. Comparison of Inactivation Doses for Tobacco Mosaic Virus and for Nucleoprotein Complex of Erythrocytes

Concentration per cent (dry weight)	Dosage necessary to reduce initial measurement to 37 per cent of initial value	
	Tobacco mosaic virus (infectivity)	Nucleoprotein complex (rigidity)
100 gr. (solid)	2.5	—
10 gr.	2.9	—
4 gr.	—	1.8 (in situ experiment)
2 gr.	2.9	—
1 gr.	—	.6 to 3.0
.4 gr.	—	.3
.1 gr.	—	.2
.02 gr.	1.5	—
.002 gr.	.5	—
1 gr. in 0.05 M glucose	—	.7
1 gr. in 2 M glucose	—	2.0
0.2+gelatine 0.07	2.4	—

Dilution effects tend to demonstrate the same phenomenon. Dilute solutions (0.05 mg. thymonucleic acid %) follow, when irradiated, an exponential decay law which can be interpreted on the basis of a superposition of two exponential curves, one representing direct and one indirect action. When the concentration is raised, the curves become less easily interpretable because some new factors appear to occur. We have tentatively explained these results by the protecting action due to split products appearing during irradiation. These would be in too small a concentration in dilute solution experiments to affect markedly the shape of the curves. The in situ experiments show that curves tend to become exponential. If the inactivation doses be considered for each concentration (inactivation being here taken to refer to the decrease of initial value of the measured magnitude for rigidity) it can be seen that the results obtained are similar to those described by Lea and collaborators (1944) for inactivation of tobacco mosaic virus. Table I shows a comparison of both series of experiments.

For concentrated solutions, as well as for the dry

virus and in the *in situ* nucleoprotein components, inactivation doses are maximum; on dilution the values observed decrease in both cases and protective effects of added substances seem to be similar.

The results for tobacco mosaic virus were interpreted on the basis of direct action when the material was irradiated in concentrated solutions or in the solid state, whereas both indirect and direct actions take place in more dilute solutions. We believe that our own experiments described above give some evidence that the same interpretation could hold for the action of radiation on nucleoprotein complexes. It would of course be extremely important to get a direct evidence of this by comparing ionic yields for different radiations. Another interesting point would be to study the effect of radiations on other macromolecular components irradiated *in situ* as compared with *in vitro* study. This could be done by studying for instance the alkaline phosphatase activity of the nucleus which is bound to a tryptophane-containing protein of the nucleus (Jeener, 1947).

We believe that the method of rigidity measurement of nucleoprotein gels will be capable of giving valuable information as to the structure and properties of the compounds studied. It would be important to obtain absolute rigidity modulus measurements and to know more of the chemical nature of the macromolecular complex studied. It would then perhaps be possible to interpret results on the basis of molecular alterations which occur during irradiation, although it does seem obvious, from data obtained on purified compounds, that long thymonucleic acid chains are split (Errera, 1946, 1947; Sparrow and Rosenfeld, 1946).

It is not possible to conclude from our experiments anything about molecular structure of the nucleoprotein complex *in situ*. Certain experiments do indeed suggest that the complex studied *in vitro* does exist in a state which has some analogies with the biological state.

One can conclude that the chemical properties altered by *in situ* irradiation are precisely those which are responsible or partly responsible for the rigid character this material takes when dispersed in the appropriate solvent.

Summary

1. We have studied radiation effects on the nucleoprotein complex of the cell nucleus, the complex being irradiated both *in situ* and *in vitro*, and studied by rigidity measurements of the complex in the gel state.

2. The *in vitro* experiments give evidence of an indirect action of the radiation. Temperature coefficients and protective effects of added substances were studied.

3. The *in situ* experiments are interpreted on the basis of a direct effect of irradiation on the same complex.

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ENZYMATIC DEGRADATION OF RIBOSENUCLEIC AND DESOXYRIBOSENUCLEIC ACIDS WITH AN ADDENDUM ON THE EFFECT OF NUCLEATES ON THE HEAT STABILITY OF PROTEINS

JESSE P. GREENSTEIN, CHARLES E. CARTER, AND HAROLD W. CHALKLEY

Because of the suggestive connection of nucleic acid with the structure of viruses and of chromosomes (*cf.* Mirsky, 1943; Gulick, 1944; Greenstein, 1944), with transmissible and inheritable changes in the morphologic pattern of certain bacteria (Avery, McLeod and McCarty, 1944), and with physiologic processes involving normal and atypical growth and development (Caspersson and Santesson, 1942), considerable contemporary interest in this class of high-molecular-weight compounds has been evoked. Knowledge of the metabolic fate of the nucleic acids within tissues is of fundamental importance in the understanding of the phenomena presumed to be elicited by these substances.

On complete hydrolysis, the nucleic acids yield nitrogenous bases (purines and pyrimidines), sugar, and phosphoric acid. These three components are combined for the most part in the form of nucleotides within the nucleic acid molecule whereby the phosphoric acid is esterified with sugar, and the base is combined in either ribosidic (yeast) or desoxyribosidic (thymus) linkage. The nucleotides are combined with each other through phosphate ester linkages, phosphoric acid thereby being doubly esterified, and these nucleotide combinations are polymerized to yield molecules of high molecular weight.

Of the nitrogenous bases, three, namely, adenine, guanine, and cytosine, contain amino groups which possess the potentiality of furnishing ammonia on hydrolytic enzymatic desamination. Of these, only guanine is desaminated in most tissues. When combined in nucleoside or nucleotide linkage, the susceptibility to enzymatic desamination of guanine, of adenine, and to a more limited extent, of cytosine, is considerably increased. Nucleotides of each of the nitrogenous bases are readily dephosphorylated in extracts of most tissues by phosphatases to which the designation of nucleotidase has been applied. The polynucleotide combinations which form the molecule of nucleic acid are held together by phosphate ester linkages which may be hydrolyzed under certain conditions by polynucleotidase, an enzyme which because of the nature of the susceptible linkage may be classed as a phosphatase. The polymerized polynucleotides are held together by forces not yet understood. Enzymes presumably responsible for the desaggregation of the nucleic acid polymers have been designated as nucleodepolymerases (*cf.* Greenstein, 1944). The designation nucleinase has frequently been applied to the enzyme complex concerned with the degradation of the intact and polymerized nucleate to the individual nucleotides. The nucleinase complex includes, therefore, those systems referred to as depolymerase and polynucleotidase.

The enzymatic desamination of the individual bases, of the nucleosides, and nucleotides, and the enzymatic dephosphorylation of the nucleotides have often been investigated (Schmidt, 1932; Conway and Cooke, 1939a and b; *cf.* Bredereck, 1938). Much less is known about the behavior under similar conditions of the more complicated molecules of the nucleic acids. Greenstein and Chalkley (1945) studied the desamination at neutral pH of ribosenucleic and desoxyribosenucleic acids added to aqueous extracts of rat spleen and noted the following: (1) The rate of desamination of the nucleates is progressive with time, reaching a maximum value of 100 micrograms of ammonia nitrogen evolved from 5 mg. of either type of nucleate; (2) although in dialized, salt-free extracts of spleen, ribosenucleic acid is desaminated to the same extent as in fresh extracts, desoxyribosenucleic acid is not at all desaminated; (3) the capacity to desaminate the desoxyribosenucleate can, however, be completely restored to the dialyzed extract by adding at 0.01 M concentration the sulfate, acetate, nitrate, or halide (except flouride) of any one of the alkali or alkaline earth metals (except beryllium), the hydrochlorides of such bases as guanidine or arginine, and the chlorides of manganese, cobalt, and nickel; (4) the sodium salts of flouride, bicarbonate, and phosphate not only fail to restore to the dialyzed extract the capacity to desaminate desoxyribosenucleate but also are inhibitory when added to the fresh extract; (5) within limits, the desamination of desoxyribosenucleate is markedly affected, that of ribosenucleate relatively little affected, by the degree of dilution of the fresh extract, a phenomenon which apparently has little to do with the level of salt concentration; (6) when dialized extracts of spleen are digested with ribosenucleate, much more phosphorus appears in dialyzable form than in fresh extracts of the tissue; and (7) the desamination of the nucleates by transplanted hepatomas is very much higher than that of normal liver.

It seems probable that the nucleates are split

into smaller fragments during the digestion period (Carter and Greenstein, 1946a), and that the ammonia may be derived from any one of these fragments, ranging from nucleic acid down to the free purine. It is not yet definitely established whether desamination precedes, accompanies, or follows the fragmentation by depolymerase, polynucleotidase, the nucleotidases, or the nucleosidases. At the present time the designation nucleodesaminase can be applied only to the progressive appearance of ammonia in digests of nucleic acid with aqueous tissue extracts, without commitment as to the size or state of the substrate at any moment. Nucleodesaminase activity may thus be the sum of the activities of numerous individual desaminases, as well as a reflection of the net activity of one or more related enzymes, such as depolymerase and polynucleotidase, etc.

The designation nucleophosphatase activity can be given to the progressive appearance of inorganic phosphate in such digests, such designation being subjected to the same limitations as mentioned previously for that of nucleodesaminase. As will be noted from the data presented, the possibility that nucleic acid or its split products can function partly as a phosphate donor makes the picture of nucleophosphatase activity still more complicated (Carter and Greenstein, 1946a).

Animal Tissues

The tissues of well over 2,000 rats and mice were employed for the present studies. The animals were killed by decapitation or cervical dislocation, and the tissues were freshly removed, ground in a glass mortar with clean sand, and then taken up in measured volumes of distilled water. After lightly centrifuging the supernatant was used as the source of enzyme. All such extracts were used within a half hour after preparation.

For experiments with dialized extracts, the appropriate freshly prepared extract was divided into halves. One-half was placed in a cellophane tube and dialized against frequently changed, distilled water for 24 hours within the ice box. The other half was kept in a glass container at the same temperature and for the same period. This fresh-tissue control after standing for 24 hours at 5° C. had very nearly the same activity as when it was newly prepared. Because of this consideration and in view of the fact that the reproducibility of the data from one tissue extract to another was as good as ± 10%, the practice of setting up this fresh-extract control was abandoned. In experiments with dialyzed extracts in which a nearly salt-free condition was desired, it was found necessary also to dialyze the nucleate solutions. Desoxyribosenucleate is prepared in the presence of sodium chloride; and since it forms an inhomogeneous mass of fibers, the possibility cannot be excluded that certain samples may contain varying quantities of this salt despite efforts at purification. As noted later completely dialyzed mixtures of various tissues with dialyzed solutions of desoxyribosenucleate yield neither ammonia nor phosphate. If dialyzed mixtures do demonstrate the presence of these substances, it is found that either extract or nucleate solution, or both, was insufficiently dialyzed.

Substrates

The nucleates employed were the soluble, purified sodium salts of yeast nucleic acid and of thymus nucleic acid. Samples of the latter type of nucleate were prepared by the method of Hammarsten (1924). The yeast nucleate gave a phosphorus value of 8.0%, the two thymus-nucleate preparations gave phosphorus values of 8.4 and 8.8%. Less than 5% of the nucleate preparations was dialyzable through cellophane.

Yeast nucleic acid serves as a model for the ribosenucleic acids and thymus nucleic acid for the desoxyribosenucleic acids. The terms "ribosenucleate" and "desoxyribosenucleate" used throughout this paper refer, therefore, to materials obtained from yeast and from the thymus gland, respectively.

Enzyme Determinations

According to the method employed, 1 cc. of the desired extract was mixed with 1 cc. of substrate. The control consisted of 1 cc. of the same extract, together with 1 cc. of distilled water. After incubation at 37° C. for a specified period, ammonia nitrogen or phosphate phosphorus was determined in both test and control mixtures, and the difference in values found gave the amount of ammonia or phosphate released from the substrate. The substrate solutions were stable in every case and by themselves never liberated ammonia or phosphate; nevertheless, such solutions were never kept for more than a few days in the refrigerator.

The pH of the mixtures was stabilized by the natural buffers of the tissues and remained within the limits of 6.8 to 6.4 during the course of the experimental period. No added buffers were employed since it was apparently unnecessary and because it was desired to note the specific effects of ions on the enzymatic systems studied, particularly in dialyzed extracts. In only one case was the pH limit mentioned exceeded, and that was when bicarbonate was used in certain experiments. The pH in such mixtures was 7.8 to 8.0. Control experiments, made with extracts to which very dilute solutions of sodium hydroxide were added so as to yield the pH 7.8 to 8.0, demonstrated very little differences in enzymatic activity from those extracts at the lower pH. It can, therefore, be assumed that whatever effects were noted with the bicarbonate ion were due specifically to the ion and not to the difference in pH from that of the digest used for comparison.

Desamination and Dephosphorylation of Purines, Nucleosides, and Nucleotides

Before investigating the enzymatic splitting of nitrogen and phosphorus from the nucleic acids, it was considered desirable to possess some information of these phenomena in digests containing the simpler components of the acids. Table 1 lists data on purines, pyrimidines, nucleosides, and nucleotides in extracts of various tissues of the rat and of the mouse.

The data given in Table 1 refer to values obtained after 5 hours of incubation. Most of the substrates listed were studied after 0.5- and 1.5-hour incubation periods, and it was noted that between 80 and 90% of the ammonia and phosphate observed at 5 hours had been split off the substrates within the first hour. There was relatively little difference between the values found after 1.5 and after 5 hours of incubation. The impression was gained that most of the reaction occurred during the first hour of incubation, went just so far, and progressed very little after that, with no marked differences in rates of desamination and dephosphorylation. Data obtained on dialyzed tissues did not vary significantly from those obtained on corresponding fresh extracts.

Table 1. Ammonia N and Phosphate P Evolved in Digests of Purines, Pyrimidines, and Nucleotides with Fresh Aqueous-tissue Extracts[1]

Tissue	Substrate											
	Adenine	Adenylic acid		Guanine	Guanosine	Guanylic acid		Cytosine[2]	Cytidine	Cytidylic acid		Uridylic acid
	N	N	P	N	N	N	P	N	N	N	P	P
Rat:												
Liver[3]	0	40	61	42	55	50	80	0	0	0	48	80
Kidney[3]	0	48	82	40	44	48	90	0	0	0	78	90
Spleen[3]	0	50	56	48	50	48	60	0	0	0	44	90
Brain	0	0	42	52	50	50	48	0	0	0	40	32
Pancreas	0	10	12	20	30	20	18	0	0	0	8	80
Muscle	0	0	0	0	40	40	24	0	0	0	0	62
Mouse:												
Liver	0	48	56	40	44	46	80	0	>0	>0	52	80
Hepatoma 587	0	48	40	44	48	48	72	0	—	0	38	90
Kidney	0	50	84	50	48	46	84	0	30	40[4]	84	90
Spleen	0	36	12	39	44	50	14	0	0	0	8	65
Brain	0	0	52	40	46	44	50	0	0	0	46	42
Pancreas	0	8	12	0	40	42	18	0	0	0	8	48
Muscle	0	0	0	0	44	40	30	0	0	0	0	22

[1] Digests consisted of 1 cc. aqueous extract (equivalent to 166 mg. tissue) plus 1 cc. of neutralized substrate solution containing, respectively, 1.24 mg. adenylic acid, 0.45 mg. adenine, 0.51 mg. guanine, 1.01 mg. guanosine, 1.28 mg. guanylic acid, 0.41 mg. cytosine, 0.85 mg. cytidine, 1.20 mg. cytidylic acid, and 1.20 mg. uridylic acid. Incubation period was 5 hours at 37° C. Results are given in terms of micrograms of ammonia N or inorganic phosphate P. Values of 50 micrograms N or 112 micrograms P are those obtained, respectively on complete desamination or dephosphorylation.

[2] Isocytosine was also resistant to enzymatic desamination.

[3] Dialyzed extracts of these tissues had very nearly the same desamination and dephosphorylation activity as did corresponding fresh extracts.

[4] Noted in mice of strains A, C, C3H, and dilute brown.

Neither adenine nor cytosine (nor isocytosine) is desaminated by any of the tissues. Of all the tissues studied, only mouse kidney, and to a very slight extent mouse liver, are capable of desaminating cytidine and cytidylic acid. The desamination of cytidylic acid is accomplished by extracts of the kidneys of A, C, C3H, and dilute brown strain mice. This species and organ specificity in regard to the desamination of cytidylic acid is indeed curious in view of the fact that all the tissues studied, except muscle, in both rats and mice, can dephosphorylate the pyrimidine nucleotide. Guanylic acid is readily desaminated, and both guanylic acid and uridylic acid are readily dephosphorylated, all seemingly to a generally wider extent than is either adenylic or cytidylic acid. The most active tissue generally is kidney, particularly that of the mouse.

Desamination and Dephosphorylation in Mixtures of Nucleotides

To some extent, as Loring and Carpenter (1943) recently showed, ribosenucleate is composed of the four nucleotides, adenylic, guanylic, cytidylic, and uridylic acids. For purposes of the present study, three sets of data were derived as follows: Desamination and dephosphorylation were studied (1) on 5 mg. ribosenucleate per cubic centimeter of substrate solution in fresh and in dialyzed tissue extracts, and (2) on equimolecular mixtures of the

four separate nucleotides in such total concentration as to be equivalent per cubic centimeter to 5 mg. ribosenucleate, *e.g.*, total weight 5.1 mg. per cubic centimeter. The data obtained were then compared (3) with the sum of the ammonia and of the phosphorus split from each of the four nucleotides digested separately and independently in each extract, each nucleotide being at the same concentration as in (2). The data are given in Table 2.

For every tissue except kidney, the summation of nucleotides is not different from that of the nucleotides mixture. In liver and in muscle extracts, the desamination of the nucleotide mixture is less than the desamination summation of the four individual nucleotides.

With the possible exception of the kidney, the dephosphorylation of the nucleates in fresh but not in dialyzed extracts falls considerably below those of the nucleotide mixture; and with the exception of both kidney and spleen, the same is true of the individual dephosphorylation of the nucleotides is the same in fresh and in dialyzed extracts and is greater than that of the nucleotide mixtures, which in turn is greater than that for ribosenucleate in fresh extracts. Except for muscle, the extent of dephosphorylation of the nucleotide mixture is very much the same as that of ribosenucleate in dialyzed tissue extracts. Like the case of each of the individual nucleotides, the desamination and dephosphorylation of the nucleotide mixture are also nearly complete during the first hour of incubation. It would appear that the splitting of phosphate when all four nucleotides are contained within a given volume is hindered, as compared with the splitting of each of the nucleotides when occupying individually the same volume. Whether each of the four nucleotides in the mixture is affected to an equal extent or whether one or more become resistant under such conditions cannot be answered at the present time. With the exception of liver and muscle, the desamination summation of the individual desamination phenomenon.

TABLE 2. COMPARISON OF AMMONIA N AND PHOSPHATE P EVOLVED IN DIGESTS OF SINGLE NUCLEOTIDES, OF EQUIMOLAR MIXTURES OF 4 DIFFERENT NUCLEOTIDES, AND OF RIBOSENUCLEATE IN FRESH AND IN DIALYZED TISSUE EXTRACTS[1]

Tissue	Ammonia N and phosphate P evolved from—							
	Summation of 4 nucleotides[2] in fresh or in dialyzed extracts		Mixture of 4 nucleotides[3] in fresh or in dialyzed extracts		Ribosenucleate[4]			
					In fresh tissue extracts		In dialyzed tissue extracts	
	N	P	N	P	N	P	N	P
Rat:								
Liver	90	269	44	106	20	55	56	124
Kidney	96	340	94	300	80	225	92	228
Spleen	98	250	90	52	96	25	96	65
Brain	50	162	50	60	32	10	42	70
Pancreas	30	118	30	60	0	0	0	78
Muscle	40	86	8	40	0	0	0	11
Mouse:								
Liver (strain A)	94	268	62	140	16	25	—	—
Hepatoma-587	96	240	60	120	50	60	—	—
Kidney	136	342	125	310	82	240	—	—

[1] Incubation period was 5 hours at 37° C. Results are given in terms of micrograms ammonia N or inorganic phosphate P; digests consisted of 1 cc. tissue extract (equivalent to 166 mg. tissue) plus 1 cc. substrate solution.

[2] Represents sum of N or P evolved from individual digests of adenylic, guanylic, cytidylic, and uridylic acids, each at concentrations given in table 1.

[3] Represents N or P evolved from equimolar mixtures of adenylic, guanylic, cytidylic, and uridylic acids, each acid at the same concentration as given in table 1. Results nearly identical in dialyzed tissues.

[4] Concentration of nucleate was 5 mg. per cubic centimeter.

It is interesting that the most active tissue in dephosphorylating individual nucleotides, mixtures of nucleotides, or the nucleates, is the kidney. Mouse kidney in particular is also the most active in desamination of the nucleotides (no doubt because of its effect on cytidylic acid), sharing with spleen the most active desaminating effect on the nucleates.

TIME COURSE OF DESAMINATION AND DEPHOSPHORYLATION OF NUCLEATES AND NUCLEOTIDE MIXTURES IN EXTRACTS OF VARIOUS TISSUES

The data at time intervals of incubation of 0.5, 1.5, 3, and 5 hours for mixture of tissue extracts with nucleates and with nucleotide mixtures are given in Figures 1-6.

EFFECT OF CONCENTRATION OF EXTRACT

The effect of two different concentrations of extract on the desamination and dephosphorylation of

ribosenucleate and of desoxyribosenucleate is shown in Table 3. In practically every case, the doubling of extract concentration (*e.g.*, from 166 to 333 mg. tissue per cubic centimeter), leaving substrate concentration constant, produces a very considerable increase in the desamination or dephosphorylation of desoxyribosenucleate and a relatively smaller increase in the case of ribosenucleate (Greenstein and Chalkley 1945). The addition of salt to the lower extract concentration causes very little effect on the splitting of nitrogen and phosphorus from desoxyribosenucleate. Changes in activity produced by changes in the concentration of extract are due in only a small measure to alterations in salt level. There seems to be no doubt that enzymatic degradation of desoxyribosenucleate is far more dependent on the degree of dilution of the extract than is that of ribosenucleate. Table 3 shows that whereas ribosenucleate in extracts of 166 mg. tissue per cubic centimeter is more susceptible than desoxyribosenucleate, in extracts of 333 mg. tissue both substrates appear to be nearly equally susceptible in all tissues studied. Thus it could be expected that on progressive dilution of the tissue extract with water, the divergence between the values for ribosenucleate and desoxyribosenucleate will increase, the desamination and dephosphorylation of the latter falling off much faster than for the former. This was actually noted in the case of all tissues studied and was pointed out earlier in the case of spleen (Greenstein and Chalkley, 1945).

The pattern of desamination and dephosphorylation activity is quite different in each tissue. Kidney has a high rate of desamination and dephosphorylation, spleen has a high rate of desamination and a very low rate of dephosphorylation, while liver has a low rate of both. Brain, muscle, and pancreas have an extremely low rate of both.

Nevertheless, there appear to be certain similarities in reaction in all the tissues; all these relate to the fact that although the dephosphorylation of the nucleates proceeds to nearly the maximum extent within the first hour and then virtually ceases thereafter, the desamination proceeds more gradually over the 5-hour interval. The desamination of the nucleotide mixture is, however, of the same relatively abrupt character shown by the dephosphorylation of the substrates studied.

TABLE 3. EFFECT OF EXTRACT CONCENTRATION ON THE DESAMINATION AND DEPHOSPHORYLATION OF RIBOSENUCLEATE AND DESOXYRIBOSENUCLEATE[1]

Tissue	Extract equivalent to 166 mg. tissue per cubic centimeter				Extract equivalent to 333 mg. tissue per cubic centimeter			
	Ribosenucleate		Desoxyribosenucleate		Ribosenucleate		Desoxyribosenucleate	
	N	P	N	P	N	P	N	P
Rat:								
Liver	25	60	5[2]	40	45	80	40	80
Fetal liver	13	53	2	10	16	121	12	35
Primary hepatoma	22	42	6	38	52	80	44	80
Kidney	85	250	64	170	90	305	85	280
Spleen	97	25	84	6	97	48	97	20
Brain	32	10	0	0	34	15	14	10
Mouse:								
Liver (strain C3H)	16	34	6	28	38	52	36	50
Hepatoma 98.15	70	60	54	60	94	68	83	68

[1] Digests consisted of 1 cc. fresh-tissue extract at concentrations designated plus 1 cc. ribosenucleate or desoxyribosenucleate at 0.5% concentration in water. Incubation period was 5 hours at 37° C. Results are given in terms of ammonia N and phosphate P.

[2] Addition of sodium chloride or magnesium sulfate at 0.01 M concentration causes only a very slight increase in activity.

Increasing the concentration of kidney extract beyond the equivalent of 333 mg. tissue per cubic centimeter produces no change in activity. The activity of extracts of transplanted hepatoma on the desamination and dephosphorylation of the nucleates is much higher than that of normal liver (Greenstein and Chalkley, 1945), but the activity of both tissues toward the nucleotide, whether singly or in the mixture, is very much the same. The high activity of the transplanted hepatoma as compared with normal liver was previously noted (Greenstein and Chalkley, 1945).

In the case of rat-kidney or rat-spleen extract at concentrations of 333 mg. tissue per cubic centimeter, the values of phosphate split from ribosenucleate and from the nucleotide mixture are very nearly the same (Figs. 2 and 4). In extracts of mouse kidney at the same concentration, the values not only of phosphate but also of ammonia split from ribosenucleate and from the nucleotide mixture are very nearly the same (Fig. 3). This interesting concordance between the related substrates in the

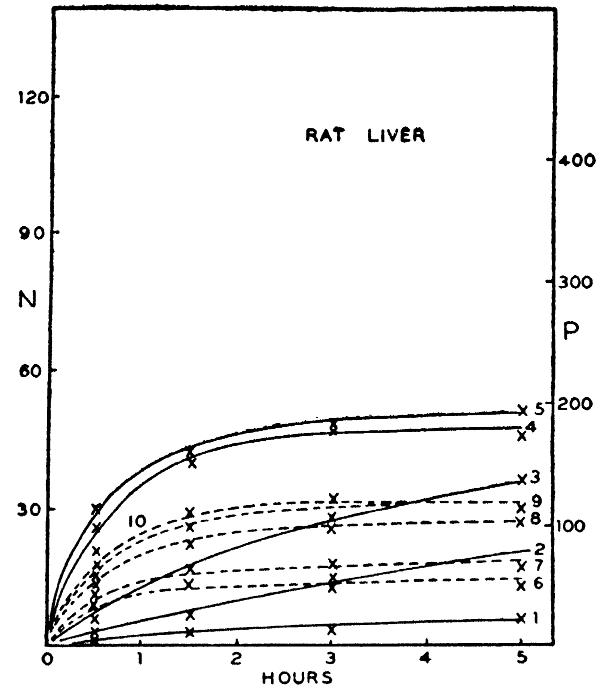

FIG. 1. Time course of the appearance of ammonia nitrogen and phosphate phosphorus in digests of nucleates or of ribose nucleotide mixtures with aqueous extracts of rat liver. Curves are as follows:

1, 2, and 4, Nitrogen from desoxyribosenucleate, ribosenucleate, and nucleotide mixture, respectively, in extracts equivalent to 166 mg. tissue per cubic centimeter;
3 and 5, Nitrogen from desoxyribosenucleate or from ribosenucleate (3) or from nucleotide mixture (5) in extracts equivalent to 333 mg. tissue per cubic centimeter;
6, 7, and 9, Phosphorus from desoxyribosenucleate, ribobosenucleate, and nucleotide mixture, respectively, in extracts equivalent to 166 mg. tissue per cubic centimeter;
8 and 10, Phosphorus from desoxyribosenucleate or from ribosenucleate (8) and from nucleotide mixture (10) in extracts equivalent to 333 mg. tissue per cubic centimeter.

Abscissa refers to hours of incubation at 37°C. Ordinates refer, respectively, to micrograms ammonia nitrogen (left side) and inorganic phosphorus (right side). Digests consisted of 1 cc. tissue extract plus 1 cc. 0.5% nucleate or 1 cc. nucleotide mixture composed of 1.24 mg. adenylic acid, 1.28 mg. guanylic acid, 1.20 mg. cytidylic acid, and 1.20 mg. uridylic acid. Continuous lines refer to evolution of ammonia nitrogen, dotted lines to evolution of inorganic phosphate phosphorus.

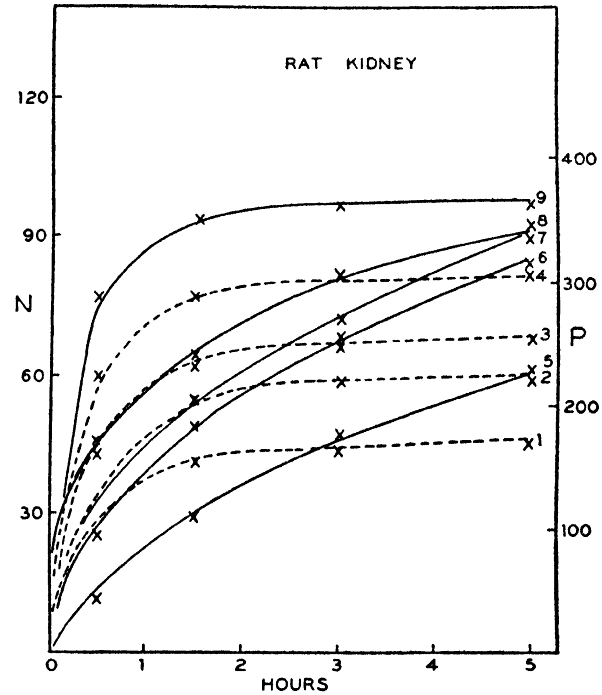

FIG. 2. Time course of the appearance of ammonia nitrogen and phosphate phosphorus in digests of nucleates or of ribose nucleotide mixtures with aqueous extracts of rat kidney. Curves are as follows:

1 and 2, Phosphorus from desoxyribosenucleate and from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;
3 and 4, Phosphorus from desoxyribosenucleate and from ribosenucleate or nucleotide mixture in extracts equivalent to 333 mg. tissue per cubic centimeter;
5 and 7, Nitrogen from desoxyribosenucleate, and from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;
6, 8, and 9, Nitrogen from desoxyribosenucleate, from ribosenucleate and from nucleotide mixture, respectively in extracts equivalent to 333 mg per cubic centimeter.

Experimental conditions and presentation of data as in Fig. 1. Data obtained with extracts of tissue equivalent to 666 mg. per cubic centimeter nearly identical with those obtained with extracts equivalent to 333 mg. per cubic centimeter.

digests described is not encountered in more dilute extracts (166 mg. tissue per cubic centimeter) of these tissues.

The similarity in activity level of the primary rat hepatoma and normal rat liver has been noted (Greenstein and Chalkley, 1946; Greenstein and Leuthardt, 1946). The transplanted rat hepatoma, like the transplanted mouse hepatoma, desaminates the nucleates to a much greater extent than does the corresponding normal liver. No data are available on the dephosphorylation rate of the transplanted rat hepatoma 31. However, the dephosphorylation activity of the transplanted mouse hepatoma, like the desamination activity, is much higher than that of liver. The desamination and dephosphorylation of the nucleates by fetal rat liver is considerably lower than that of the adult liver, but one interesting fact may be noted. The dephosphorylation of ribosenucleate and of desoxyribosenucleate is not very different in the adult liver whereas in the fetal liver the dephosphorylation of the former far

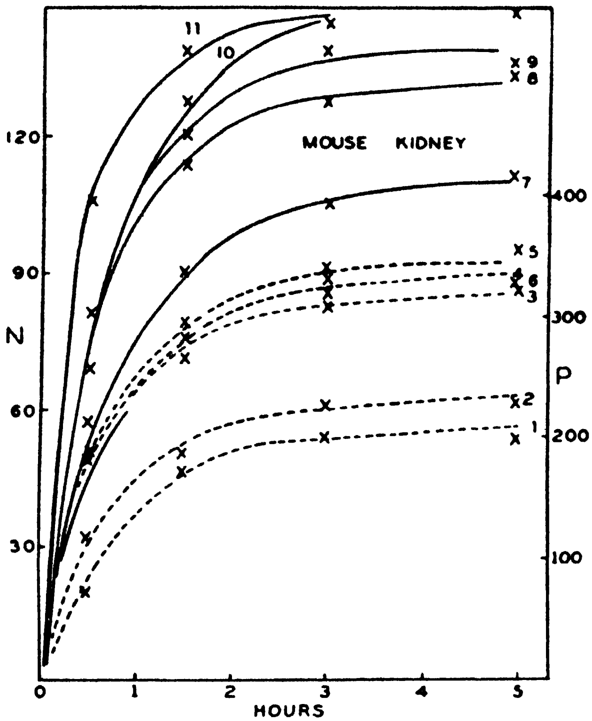

FIG. 3. Time course of the appearance of ammonia, nitrogen and phosphate phosphorus in digests of nucleates or of ribose nucleotide mixtures with aqueous extracts of mouse kidney. Curves are as follows:

1, 2, and 3, Phosphorus from desoxyribosenucleate from ribosenucleate, and from nucleotide mixture, respectively, in extracts equivalent to 166 mg. tissue per cubic centimeter;

4 and 5, Phosphorus from desoxyribosenucleate and from either ribosenucleate or nucleotide mixture in extracts equivalent to 333 mg. tissue per cubic centimeter;

6, 7, and 10, Nitrogen from desoxyribosenucleate, from ribosenucleate, and from nucleotide mixture, respectively, in extracts equivalent to 166 mg. tissue per cubic centimeter;

8 and 11, Nitrogen from desoxyribosenucleate and from nucleotide mixture in extracts equivalent to 333 mg. tissue per cubic centimeter;

9, Nitrogen from ribosenucleate and from nucleotide mixture in extracts equivalent to 333 mg. tissue per cubic centimeter, and from nucleotide mixture in extracts equivalent to 166 mg. tissue per cubic centimeter.

Experimental conditions and presentation of data as in Fig. 1. Data obtained with extracts of tissue equivalent to 666 mg. of tissue per cubic centimeter nearly identical with those obtained with extracts equivalent to 333 mg. per cubic centimeter.

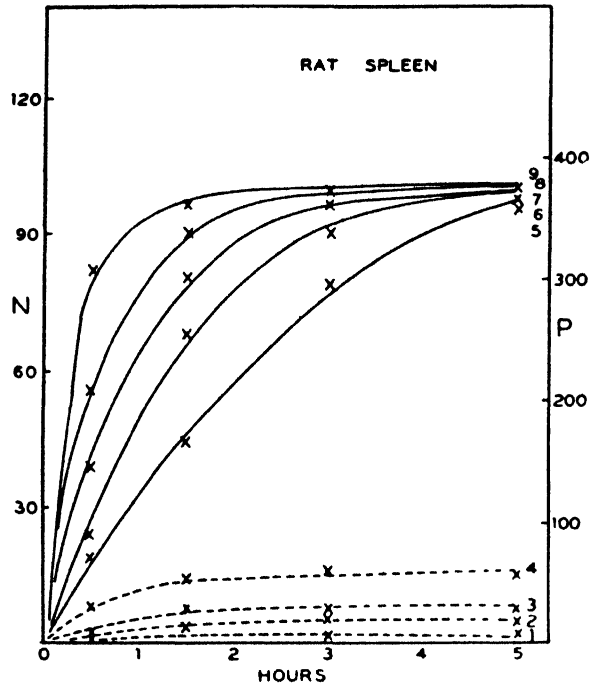

FIG. 4. Time course of the appearance of ammonia nitrogen and phosphate phosphorus in digests of nucleates or of ribose nucleotide mixtures with aqueous extracts of rat spleen. Curves are as follows:

1 and 3, Phosphorus from desoxyribosenucleate or from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;

2 and 4, Phosphorus from desoxyribosenucleate and from ribosenucleate or nucleotide mixture in extracts equivalent to 333 mg. tissue per cubic centimeter;

5 and 7, Nitrogen from desoxyribosenucleate and from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;

6, 8, and 9, Nitrogen from desoxyribosenucleate, from ribosenucleate, and from nucleotide mixtures, respectively, in extracts equivalent to 333 mg. tissue per cubic centimeter.

Experimental conditions and presentation of data as in Fig. 1.

exceeds that of the latter. Indeed at the higher extract concentration, the dephosphorylation of ribosenucleate in fetal liver exceeds that in adult liver.

Effect of Salts and of Dialysis

It was repeatedly noted in earlier experiments that dialyzed spleen extracts split off more dialyzable phosphorus from ribosenucleate, but no more ammonia nitrogen, than did fresh extracts of this tissue (Greenstein and Chalkley, 1945). Under these conditions, dialyzed spleen extract split neither phosphorus nor ammonia from desoxyribosenucleate. The addition of any one of a wide variety of salts to the dialyzed spleen extract restored the desamination capacity to act on desoxyribosenucleate, but no further tests were made for phosphorus in the presence of this substrate.

The effect of dialysis and the effect of the addition of a variety of salts to both dialyzed and to fresh extracts of a number of rat tissues are given in Table 4.

The following points in Table 4 may be noted: (1) Tissue extracts require salts for the metabolism

of desoxyribosenucleate but not for that of ribosenucleate; (2) except in extracts of kidney, sodium bicarbonate and sodium fluoride have an inhibiting effect on the desamination and dephosphorylation of the nucleates whether in fresh or in dialyzed extracts; (3) dialyzed extracts of liver, spleen, brain, pancreas, and muscle produce more dephosphorylation of ribosenucleate than do fresh extracts of these tissues, while dialyzed extracts of liver and of brain produce more desamination of this substrate; (4) dialyzed extracts of kidney plus salts produce more desamination of desoxyribosenucleate but no more dephosphorylation than do fresh extracts; (5) dialyzed extracts of all the tissues neither desaminate nor dephosphorylate desoxyribosenucleate; (6) addition of the chlorides of sodium, potassium, calcium, magnesium, or arginine considerably enhances, beyond that of the fresh tissue, the capacity of the dialyzed extracts of liver to desaminate and dephosphorylate desoxyribosenucleate, and of spleen, brain, and pancreas to dephosphorylate this substrate; (7) addition of bicarbonate or fluoride to the dialyzed extracts of the tissues fails to restore either desamination or dephosphorylation capacity for desoxyribosenucleate; (8) all the effective chlorides appear to be nearly equally active in the degree to which each restores or enhances, beyond that of the fresh tissue, the desamination and dephosphorylation capacity of the dialyzed tissues; and (9) although in the fresh-tissue extract the dephosphorylation of ribosenucleate exceeds that of desoxyribosenucleate, the reverse is true in dialyzed extracts of liver and of spleen (in the presence of added salts).

These findings are generally consistent with those noted earlier on spleen (Greenstein and Chalkley, 1945) and by extension of the investigation to still other tissues have emphasized the phenomena. The considerably greater desamination and dephosphorylation capacity of dialyzed extracts of certain tissues for ribosenucleate and for desoxyribosenucleate (when salt is present) is noteworthy, particularly in view of the fact that the desamination and dephosphorylation of the individual nucleotides or of the equimolecular nucleotide mixture are practically the same in fresh and dialyzed extracts. These observations of fresh extracts and on dialyzed extracts in the presence and absence of salts have been repeatedly confirmed.

TABLE 4. EFFECT OF SALTS ON THE DESAMINATION AND DEPHOSPHORYLATION OF NUCLEATES IN FRESH AND IN DIALYZED RAT-TISSUE EXTRACTS[1]

Substrate[2]	Salt[3]	Liver				Kidney				Spleen				Brain				Pancreas				Muscle			
		Fresh		Dialyzed		Fresh		Dialyzed		Fresh		Dialyzed		Fresh		Dialyzed		Fresh		Dialyzed		Fresh		Dialyzed	
		N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Ribosenucleate	—	23	50	56	124	86	240	92	228	92	20	96	65	32	10	42	70	0	0	0	78	0	0	0	11
	$MgSO_4$	26	50	60	130	84	236	92	230	92	20	96	65	—	—	—	—	—	—	—	—	—	—	—	—
	$NaHCO_3$	7	40	10	14	74	250	88	240	43	10	14	18	—	—	—	—	—	—	—	—	—	—	—	—
	NaF	7	32	0	10	62	248	40	140	28	10	0	8	—	—	—	—	—	—	—	—	—	—	—	—
Desoxyribose-nucleate	—	5	40	0	0	58	180	0	0	80	6	5	4	0	0	0	0	0	0	0	32	0	0	0	0
	NaCl	6	40	20	110	58	200	102	210	80	6	86	160	0	5	0	25	0	5	0	32	0	0	0	0
	KCl	6	40	20	112	58	210	102	200	80	6	86	150	—	—	—	—	—	—	—	—	—	—	—	—
	$CaCl_2$	6	42	22	124	58	208	102	220	80	6	84	180	—	—	—	—	—	—	—	—	—	—	—	—
	$MgCl_2$	6	50	24	156	58	220	104	220	84	8	90	210	—	—	—	—	—	—	—	—	—	—	—	—
	Arginine HCl	6	45	20	120	58	210	100	195	86	6	92	200	—	—	—	—	—	—	—	—	—	—	—	—
	$NaHCO_3$	2	35	0	8	20	200	0	0	10	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaF	0	0	0	0	15	210	12	0	0	2	0	4	—	—	—	—	—	—	—	—	—	—	—	—

[1] Digests consisted of 1 cc. tissue extract(equivalent to 166 mg. tissue) plus 1 cc substrate. Extracts were dizalyed for 24 hours against distilled water at 5 C°. Salts added as 0.2 cc. of 0.15 M stock solutions to extracts prior to mixing with substrate. Incubation period was 5 hours at 37° C. Results are given in terms of micrograms ammonia N and phosphate P.

[2] Substrates at concentration of 5 mg. per cubic centimeter water. Substrate solutions used with dialyzed extracts were themselves dialyzed against water for 24 hours.

[3] In final concentration of 0.014 M in digests. Salts at concentrations higher than 0.5 M were inhibitory.

The requirement of the tissue extracts for the presence of salt in order to desaminate and to dephosphorylate desoxyribosenucleate (as contrasted with the apparent dispensability of salts in the desamination and dephosphorylation of ribosenucleate) can apparently be satisfied to an equal extent by a wide variety of monovalent and divalent salts. Experiments on the time course of the desamination of desoxyribosenucleate by dialyzed rat-spleen extract showed that the rate of reaction was very nearly the same in the presence of 0.01 M sodium chloride, magnesium chloride, or arginine monohydrochloride.

The inhibitory effect of bicarbonate and of fluoride on the enzymatic capacities of the tissues for the nucleates is also observed to a smaller extent in the case of the individual nucleotides. The interesting fact in regard to these particular ions in their relation to the enzymatic degradation of the

nucleates is not only that they inhibit activity in the fresh extract but also that they fail to restore the activity of the dialyzed extract.

TABLE 5. EFFECT OF SALTS OF VARIOUS KINDS ON RESTORATION OF CAPACITY FOR DESAMINATING AND DEPHOSPHORYLATING DESOXYRIBOSENUCLEATE IN DIALYZED RAT-SPLEEN EXTRACT[1]

Salt	Ammonia N	Phosphate P
	Micrograms	Micrograms
No salt	[2]0	[3]2
Sodium fluoride	0	0
Sodium chloride	90	150
Sodium bromide	92	158
Sodium iodide	90	145
Sodium nitrate	90	160
Sodium sulfate	88	160
Sodium citrate	90	120
Sodium succinate	88	140
Sodium glutamate	89	200
Sodium acetate	86	180
Sodium nitroprusside	90	180
Sodium bicarbonate	0	0
Lithium chloride	92	190
Potassium chloride	90	185
Rubidium chloride	94	180
Cesium chloride	90	172
Beryllium sulfate	0	0
Magnesium chloride	90	210
Calcium chloride	94	200
Strontium chloride	92	190
Barium chloride	90	190
Manganese chloride	88	200
Nickel chloride	80	180
Cobalt chloride	82	180
Guanidine hydrochloride	94	190
Arginine hydrochloride	92	190

[1] Digests and experimental conditions as in Table 4.
[2] Value for fresh extract, 84 micrograms ammonia N.
[3] Value for fresh extract, 6 micrograms inorganic phosphate P.

Earlier work (Greenstein and Chalkley, 1945) and the data in Table 4 indicate that dialysis of a tissue extract results in a loss in the capacity of the extract to desaminate and to dephosphorylate desoxyribosenucleate. This capacity can be restored by the various salts listed (Table 4). In the study on dialyzed spleen extracts (Greenstein and Chalkley, 1945), a wide range of salts, many of them not generally encountered physiologically, possessed the capacity of restoring to such extracts the ability to desaminate desoxyribosenucleate. In order to see whether the same salts also restore the dephosphorylating ability to such extracts, an investigation similar to those described was made. The data are given in Table 5. The desamination data are from the earlier report (Greenstein and Chalkley, 1945).

It may be noted (1) that those salts effective in restoring to the dialyzed extract the ability to desaminate desoxyribosenucleate are also effective in restoring the dephosphorylation ability, and (2) that those salts that inhibit the restoration of the desaminating capacity also inhibit the restoration of the dephosphorylating capacity. The data in Table 5 simply further emphasize the data in Table 4, but the former show that even nonphysiologic ions possess the ability to restore to the dialyzed extract the ability to metabolize desoxyribosenucleic acid.

ACID-SOLUBLE PHOSPHORUS IN DIGESTS OF NUCLEATES IN FRESH AND DIALYZED EXTRACTS

The data in Table 6 show (1) that under otherwise similar conditions the total acid-soluble phosphorus formed from the nucleates in fresh extracts of rat spleen appears to be higher in the case of desoxyribosenucleate than of ribosenucleate, and in the case of the former substrate, higher in dialyzed,

TABLE 6. ACID-SOLUBLE PHOSPHORUS FORMED IN DIGESTS OF NUCLEATES IN FRESH AND DIALYZED EXTRACTS OF RAT SPLEEN[1]

Substrate[2]	Salt[3]	Total acid-soluble P		Corrected acid-soluble P[4]	
		Fresh	Dialyzed	Fresh	Dialyzed
Ribosenucleate	—	23	—	3	—
	$NaHCO_3$	23	20	13	2
Desoxyribosenucleate	—	76	8	70	4
	NaCl	100	170	94	10
	$McCl_2$	100	214	92	4
	Arginine HCl	94	210	88	10
	$NaHCO_3$	15	0	12	0

[1] Digests consisted of 1 cc. extract (equivalent to 166 mg. tissue) plus 1 cc. substrate. Extracts were dialyzed for 24 hours against distilled water at 5° C. Salts added as 0.2 cc. of 0.15 M stock solutions to extracts prior to mixing with substrate. Incubation period was 5 hours at 37° C. At end of incubation period, 1 cc. of 5% trichloracetic acid was added to each tube, the mixture centrifuged, and total P determined in supernatant. Results are given in terms of micrograms P.
[2] At 5 mg. per cubic centimeter water.
[3] In final concentration of 0.014 M in digests.
[4] Total acid-soluble P minus inorganic phosphate P. See Table 4.

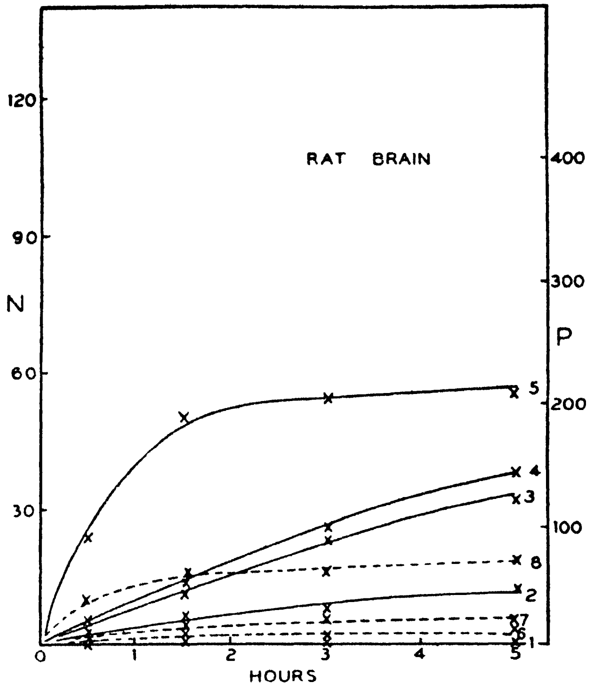

FIG. 5. Time course of the appearance of ammonia nitrogen and phosphate phosphorus in digests of nucleates or of ribose nucleotide mixtures with aqueous extracts of rat brain. Curves are as follows:

1, Nitrogen or phosphorus from desoxyribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;
3, Nitrogen from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter;
2, 4, and 5, Nitrogen from desoxyribosenucleate, from ribosenucleate, and from nucleotide mixtures, respectively, in extracts equivalent to 333 mg. tissue per cubic centimeter;
6, 7, 8, Phosphorus from desoxyribosenucleate, from ribosenucleate, and from nucleotide mixture, respectively, in extracts equivalent to 333 mg. tissue per cubic centimeter. Curve 6 also refers to phosphorus from ribosenucleate in extracts equivalent to 166 mg. tissue per cubic centimeter.

Experimental conditions and presentation of data as in Fig. 1.

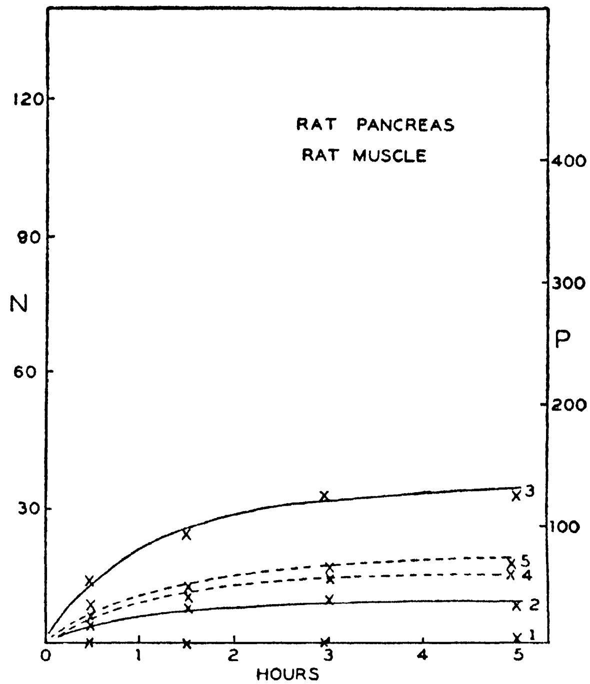

FIG. 6. Time course of the appearance of ammonia nitrogen and phosphate phosphorus in digests of nucleate or of ribose nucleotide mixtures with aqueous extracts of rat pancreas or muscle. Curves are as follows:

1, Nitrogen or phosphorus from ribosenucleate and desoxyribosenucleate in extracts of pancreas or muscle at concentrations of tissue equivalent either to 166 or to 333 mg. per cubic centimeter;
2 and 3, Nitrogen from nucleotide mixtures in extract of muscle and in extract of pancreas at concentrations of tissue equivalent to 333 mg. per cubic centimeter;
4 and 5, Phosphorus from nucleotide mixtures in extract of muscle and in extract of pancreas at concentrations of tissue equivalent to 333 mg. per cubic centimeter.

Experimental conditions and presentation of data as in Fig. 1.

salt-containing extracts than in fresh extracts, (2) when this total acid-soluble phosphorus is corrected for by the amount of inorganic phosphate phosphorus present, the organically bound, acid-soluble phosphorus in the fresh tissue is still higher in the case of desoxyribosenucleate as compared with ribosenucleate, (3) in the dialyzed, salt-containing tissue relatively little organically bound, acid-soluble phosphorus is present, by far the greater proportion of the total acid-soluble phosphorus being inorganic phosphate, and (4) dialysis of the extract or addition of bicarbonate has a decidedly inhibitory effect on the formation of both inorganic and organically bound, acid-soluble phosphorus. Comparison of the last two columns in Table 6 suggests that the organically bound, acid-soluble phosphorus derived from the nucleates is split further in the dialyzed, salt-containing extract into inorganic phosphate. Thus the considerable rise in the amount of inorganic phosphate in digests of dialyzed extract with desoxyribosenucleate and salt is caused to a large extent by the splitting into inorganic phosphate of the acid-soluble, organically bound phosphorus produced from the nucleate. Not all the rise in inorganic phosphate level is accounted for in this way, and part of this rise must have its origin in other sources, perhaps in the phosphorus of the acid-insoluble fraction. In the absence of added salt, little total acid-soluble phosphorus appears in the dialyzed extract. The effect of added salts may be to activate the formation of organically bound, acid-soluble phosphorus from which the inorganic phosphate is derived.

DESOXYRIBOSENUCLEODEPOLYMERASE ACTIVITY

There is an enzyme present in tissues which catalytically effects a reduction in the extreme asymmetric shape of sodium desoxyribosenucleate (Greenstein and Jenrette, 1941). The activity of this enzyme can be conveniently followed by examining over the time interval of incubation the changes in those physical properties of the nucleate which are the result of changes in its shape. Observation of the structural viscosity of solutions of the nucleate in ranges of velocity gradients where the viscosity changes are notably sensitive to changes in molecular asymmetry affords an excellent method of following the activity of the responsible enzyme system. Because the activity of the enzyme is measured by a physical property of the substrate which presumably is based upon a special state of molecular aggregation, the designation depolymerase was given to the enzyme, without commitment as to the mechanisms involved in its action or to the nature of the susceptible linkages within the substrate. In mixtures of tissue extracts with desoxyribosenucleate, the depolymerization of the substrate may be considered to be the primary step in its degradation. It is of some interest to see whether it bears any relation to subsequent desamination and dephosphorylation reactions induced by other enzyme systems in the extract.

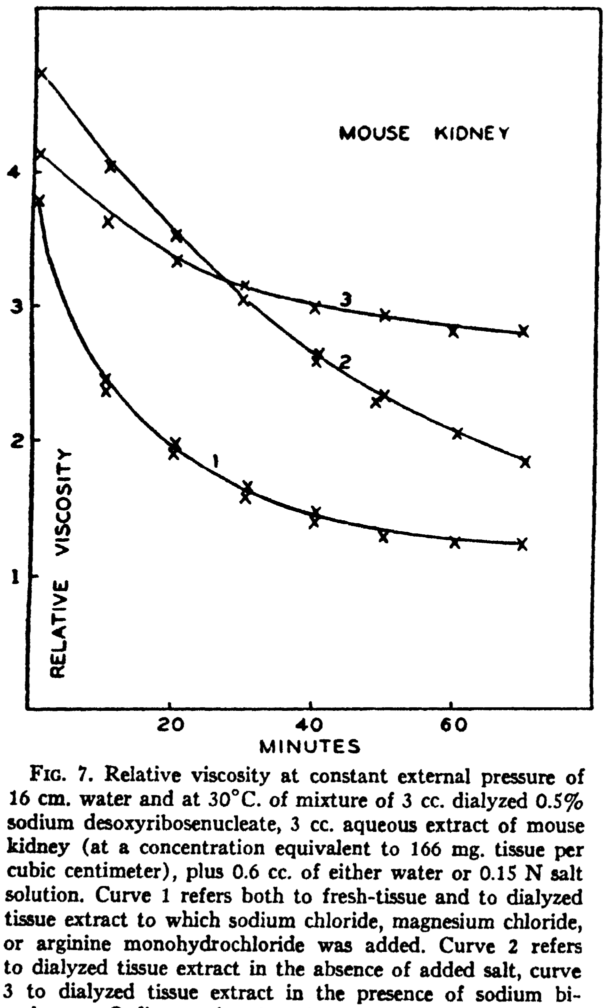

FIG. 7. Relative viscosity at constant external pressure of 16 cm. water and at 30°C. of mixture of 3 cc. dialyzed 0.5% sodium desoxyribosenucleate, 3 cc. aqueous extract of mouse kidney (at a concentration equivalent to 166 mg. tissue per cubic centimeter), plus 0.6 cc. of either water or 0.15 N salt solution. Curve 1 refers both to fresh-tissue and to dialyzed tissue extract to which sodium chloride, magnesium chloride, or arginine monohydrochloride was added. Curve 2 refers to dialyzed tissue extract in the absence of added salt, curve 3 to dialyzed tissue extract in the presence of sodium bicarbonate. Ordinate, viscosity of mixtures relative to that of the extract; abscissa, period of incubation of the mixtures in the viscometer. Bingham-Jackson viscometers were employed.

As noted in Tables 4 and 5, dialysis of the extracts removes their capacity to desaminate and to dephosphorylate desoxyribosenucleate, but on addition of certain salts to the dialyzed extract this capacity can be restored. Although numerous salts are included among these restorative salts, sodium bicarbonate and sodium fluoride are not (Table 4). In order to note the activity of the depolymerase for sodium desoxyribosenucleate under these conditions, viscosimetric studies of various mixtures incubated in the viscometer were made. They are illustrated in Figure 7.

Figure 7 shows that the progressive, enzymatic depolymerization of the nucleate is slowed in the dialyzed extract as compared with the fresh extract, but it apparently is not prevented. Addition of sodium chloride, magnesium chloride, or arginine monohydrochloride in final concentration of 0.015 N restores the activity of the depolymerase to that found in the fresh extract. Under conditions whereby desamination and dephosphorylation of the substrate are completely prevented, namely, in the salt-free, dialyzed extract, depolymerization of the substrate can, nevertheless, take place, even if at a somewhat slower rate than that in the fresh extract. It may be that the inert, nonenzymatic proteins in the dialyzed extract exert a weak, activating effect on the depolymerase, an effect considerably augmented when certain inorganic or organic salts are added. It is apparently possible to have some depolymerase activity in the absence of any desamination or dephosphorylation. It may therefore be assumed that the substrate aggregates of lower asymmetry, formed as a result of the action of depolymerase, do not yield ammonia or phosphate in the absence of salt and that the presence of such salt is even more crucial to those subsequently acting systems which produce organically bound, acid-soluble phosphorus (Table 5) and perhaps to the activity of the desaminases and dephosphorylases than to the activity of the depolymerase. Bicarbonate added to the dialyzed tissue extracts does not restore the capacity of such extract to desaminate or to dephosphorylate desoxyribosenucleate; and as noted in Fig. 7, it inhibits the depolymerase in the dialyzed extract.

Sodium chloride, magnesium chloride, and arginine monohydrochloride are equally effective in restoring the full capacity of the dialyzed extract

to depolymerize the desoxyribosenucleate (Fig. 7) as well as in producing in such digests organically bound, acid-soluble phosphorus and in freeing ammonia and phosphate. This catholicity in effect by so many diverse salts is remarkable. The fact that the restorative effects of certain salts appear to be connected with so many phenomena involved in the metabolism of desoxyribosenucleate suggests either that these effects are specific at one crucial stage in the early break-down of this substrate or that the degradation products bear some family resemblance among themselves in their reaction to the presence of such salts.

Resumé

The most obvious thing to note in the studies herein described is the fact that extracts of different tissues affect the desamination and dephosphorylation of the nucleates to different extents. On the one hand there is the kidney which desaminates and dephosphorylates the nucleates to a very large extent, and on the other hand there are pancreas and muscle which do not affect these substrates to any appreciable extent. Between these extremes there are spleen, which desaminates considerably and dephosphorylates very little, liver, which both desaminates and dephosphorylates relatively little, and brain, which affects only ribosenucleate and that to a very slight extent. Yet even the tissues ineffective on the nucleates, *e.g.*, muscle, pancreas, and brain, desaminate and dephosphorylate the free nucleotides, susceptible purines, and nucleosides, and nucleotide mixtures, to a relatively smaller extent than do the more active tissues it is true. It may be that extracts of muscle, brain, etc., do not degrade the nucleates to particles the size of susceptible free nucleotides. On the whole, it appears probable that the nucleotide phosphatases and nucleotide desaminases are not generally active on the nucleates or their early degradation products and are distinct from those systems responsible for the desamination and dephosphorylation of the nucleates or their immediate split products.

After dialysis, and in the presence of salts, extracts of liver desaminate and dephosphorylate the nucleates more than do fresh extracts; extracts of spleen, pancreas, and brain dephosphorylate the nucleates more than do fresh extracts, while extracts of kidney do not desaminate or dephosphorylate the nucleates any more than do the fresh extracts of this tissue. The enormous increase in the inorganic products in digests of desoxyribosenucleate with dialyzed tissues takes place only in the presence of a wide variety of salts and does not occur either in the absence of salt or in the presence of such salts as fluoride or bicarbonate. The reason why dialysis does not increase the desamination and dephosphorylation of nucleates in extracts of kidney or the desamination of nucleates in the extracts of spleen may be due to the possibility that these substrates are already desaminated or desphosphorylated to the nearly maximum extent in the respective fresh tissues. Apparently when in the fresh tissue the desamination proceeds to the extent of 100 micrograms and the dephosphorylation to 200-300 micrograms per 5 mg. of nucleate, dialysis of the tissuue causes no augmentation of enzymatic degradation of ribosenucleate; in the case of desoxyribosenucleate in the presence of effective salt, only a restoration of the degradation takes place to an extent characteristic of the fresh tissue. In extracts of liver, where the desamination and dephosphorylation capacity is originally low, and in spleen, where dephosphorylation is initially low, it is possible by dialysis and salt treatment procedures to enhance the desamination and dephosphorylation above that observed in the respective fresh tissues.

It would appear that something is removed from extracts of liver, spleen, brain, etc., on dialysis, which permits the further appearance of inorganic phosphate and ammonia and which, conversely, in the fresh extract inhibits in some way the appearance of these products. In such tissues as designated, dialysis does not apparently affect the desamination and dephosphorylation of simple nucleotides or of mixtures of nucleotides.

There are two possible explanations as to the differences observed in certain tissues in the fresh and in the dialyzed state. One may be based upon the assumption that in the fresh extract of liver or spleen there is some inhibitor for dephosphorylation and desamination which is removed by dialysis. The second explanation may be based upon the assumption that there is some acceptor for phosphate and for ammonia which is present in fresh extracts of liver or of spleen and which likewise is lost on dialysis. According to the latter explanation, the nucleic acids or their larger split products but not the nucleotides may function as phosphate and ammonia donors. To what recipients this phosphate may be donated is not known. According to either explanation, extracts of kidney possess neither an inhibitor for dephosphorylation nor a system capable of accepting phosphate from nucleic acid.

The data in Table 6 indicate that the increase in inorganic phosphate in the dialyzed extracts is at the expense of the organically bound, acid-soluble phosphorus formed in the digest of nucleic acid. For this purpose, in the case of desoxyribosenucleate the presence of salts is required. Sodium chloride, magnesium chloride, and arginine monohydrochloride are equally effective in this respect, whereas sodium bicarbonate is toxic. It would appear as if salt were essential under these circumstances in order to degrade the desoxyribosenucleate into acid-soluble fractions which in the dialyzed but salt-treated extract break down further to yield inorganic phosphate.

Bicarbonate does not inhibit the dephosphorylation of either ribosenucleate or desoxyribosenucleate in fresh extracts of kidney, or the dephosphorylation of ribosenculeate in dialyzed kidney extract. It does not restore the dephosphorylation of desoxyribosenucleate in the dialyzed extract of kidney. If there were an inhibitor for dephosphorylation of nucleates in fresh extracts of other tissues, it would be difficult to believe that it could be solely bicarbonate.

The fact that the amount of phosphate phosphorus is higher in dialyzed extracts of liver and of spleen indicates that the enzymes responsible for the degradation of the nucleates in these tissues are not necessarily weaker than those in the kidney, but simply that conditions in the fresh extracts of the two former tissues are such that except by dialysis the full potentiality for the break-down of the nucleates in such tissues is not realized. Whether such conditions in the fresh tissue consist of the presence of an inhibitory, interfering system or of an accepting system for phosphate cannot be definitely answered at the present time. Further research is needed to elucidate this point.

The comparison in rates of desamination and dephosphorylation of individual nucleotides, of nucleotide mixtures, and of the nucleates, each group at equivalent concentration (Table 2), suggests that the lowered values in the case of the mixture of nucleotides as compared with the summation of the individual nucleotides are the result of competitive inhibition in digests involving the nucleotide mixture. The ribosenucleate incubated in fresh tissue extracts yields ammonia and phosphate in even lower amount than does the nucleotide mixture but the values found in nucleate digests with dialyzed tissues approximate those noted for the nucleotide mixture. In more concentrated tissue extracts of kidney and spleen, the dephosphorylation of ribosenucleate and of the nucleotide mixtures is very nearly the same. In dialyzed extracts of liver, kidney, and spleen, ribosenucleate acts as if it were a mixture of four ribosenucleotides in equivalent proportions. There is no difference in desamination and dephosphorylation of the ribosenucleotide mixture in fresh or in dialyzed extracts, but there is in the case of the ribosenucleate. Whatever interferes in the fresh extract with the desamination and dephosphorylation of ribosenucleate must act upon the latter in a stage higher than that of the mononucleotide.

The very considerable differences in conditions necessary to derive ammonia and phosphate from desoxyribosenucleate as compared with ribosenucleate are shown by the data in Tables 4 and 6. The wide variety of salts effective in the degradation of desoxyribosenucleate is particularly impressive and as contrasted with ribosenucleate offers the possibility of regulating the metabolism of the desoxyribosenucleate by regulating the salt level. There is apparently little specificity among the effective salts, monovalent metal cations being as effective as monovalent organic cations and divalent metal cations. The ions need not even be commonly physiologic, for as earlier shown (Greenstein and Chalkley, 1945, and Table 5) such ions as lithium, cesium, rubidium, strontium, and barium were as active as sodium, magnesium, or calcium, while sodium glutamate was as active as arginine hydrochloride. The fact that the desamination and dephosphorylation of desoxyribosenucleate is strongly influenced also by the concentration of the extract (Table 3) suggests that the nonenzymatic proteins of the extract may also exert an activating influence on the metabolism of this substrate. The dialyzed tissue extract is still capable of desaminating and dephosphorylating ribosenucleate but loses the capacity to affect desoxyribosenucleate. The capacity to restore, and in some tissues to exceed the normal level of desaminating and dephosphorylating desoxyribosenucleate can be effected by the wide variety of salts mentioned. It seems probable, therefore, that although desamination and dephosphorylation may not be directly related in the fresh extract, they may be indirectly related by virtue of being mutually dependent upon a common state of the desoxyribosenucleate arrived at in the presence of effective salt. Furthermore, the fact that the same wide range of salts, some physiologic and some nonphysiologic, some inorganic and others organic, can restore to the dialyzed extract the capacities for both desaminating and dephosphorylating desoxyribosenucleate, and the further fact that the same salts (fluoride, bicarbonate) do not restore the capacity for either desaminating or dephosphorylating suggests that the salt effect is concerned with the activation of the enzymatic degradation of the nucleate to a form that can be both desaminated and dephosphorylated. The possibility must be envisaged that the appearance of ammonia and inorganic phosphate in the digests described may be secondary to that enzymatic degradation of the nucleates for which any one of an extensive variety of salts is required. It is difficult to see how or why so many diverse salts affect in equal manner such different processes as desamination and dephosphorylation. Nevertheless, the problem at what precise point or points in the enzymatic breakdown of desoxyribosenucleate the need for salts occurs remains for future investigation.

The inhibitory effect of the bicarbonate and of the fluoride ions is difficult to understand, for these ions not only are inhibitory in the fresh extract but also are nonrestorative in the dialyzed extract. It is possible that they either participate in competing reactions or lack the necessary dimensions to combine effectively with the specific enzyme protein in order to activate it.

Fresh extracts of the various tissues show very decided differences among themselves in their ca-

pacity to desaminate and dephosphorylate the two types of nucleates, kidney being by far the most active. Since in the dialyzed extracts of liver and spleen, the amount of phosphate observed tends to increase and approach the value noted in the kidney extracts, it would appear that the differences between such tissues as liver, spleen, and kidney tend to diminish when the extracts of such tissues are dialyzed and then treated with effective salts (Table 4). This observation is remindful of that made by Chalkley and Greenstein (1945) in the determination of the decolorization rates of methylene blue in fresh and dialyzed extracts of liver and of hepatoma. The rates of decolorization of the dye in the presence of nucleates were found to be different in fresh extracts of the two tissues but were nearly the same in dialyzed extracts of these tissues. It might appear that the differences in nucleic acid metabolism noted in fresh extracts of various tissues are due for the most part to dialyzable components in these tissues, and that when such components are removed by dialysis, the activity values for the various tissues tend to approach each other. These findings emphasize the fact that when one measures enzymatic activity in a fresh-tissue extract or homogenate, what is being measured is not necessarily the true activity or the concentration of the enzyme but the net effect of all conditions present in the material being studied. The limiting factor under these conditions may not be the enzyme itself, and the result of measuring certain products of the reaction may be simply to yield a reflection of concomitant and sometimes competing reactions and thus no exact picture of the primary reaction.

In fresh-tissue extracts, the desamination and dephosphorylation of ribosenucleate invariably exceeded that of desoxyribosenucleate. In dialyzed extracts of liver and of spleen and in the presence of effective salts, the level of dephosphorylation of desoxyribosenucleate exceeded that of ribosenucleate.

There seems to be little doubt that during the course of the digestion of either type of nucleate in tissue extracts degradation of the nucleate takes place which leads to fragments sufficiently small to pass through a cellophane membrane (less than 10,000 in molecular weight based on a spherical molecule). At what stage in the fragmentation desamination and dephosphorylation begin is not yet known. From the data in the present paper, it seems probable that the enzymes concerned with the desamination and dephosphorylation of the nucleotides may not be the same as those concerned with the production of ammonia and inorganic phosphate from the nucleic acids or presumably their higher split products. An analogy may be drawn from the metabolism of proteins in which a complex of proteases and peptidases is concerned, each enzyme acting in a specific manner upon the substrates of different size and configuration. An entire range of polypeptides of different sizes can apparently be attacked by such a complex proteolytic system as crude trypsin, and the designation of such terms as "aminopeptidase" and "carboxypeptidase" refers not to the size of the polypeptide substrate but to the point of attack by the enzyme. The rate-limiting factor in the breakdown of protein by the crude proteolytic system would be the activity of the primary protease reaction, but this in turn would be influenced by the rate at which the products of the reaction were removed. Without belaboring the analogy unduly, it may be hypothesized that some similar complex mechanism is operative in the case of the enzymatic degradation of the nucleic acids, fragmentation, desamination, and dephosphorylation taking place alternately and concurrently as the digestion proceeds.

The maximum inorganic phosphate split from ribosenucleate in extracts of rat kidney amounts to 60-80% of the total nucleate phosphate. The ammonia split under the same conditions in extracts of rat kidney or spleen amounts to 60-70% of the total amino nitrogen in the ribosenucleate, and in extracts of mouse kidney to 90% of the total amino nitrogen (Figs. 2-4). The difference in desamination in extracts of rat or mouse kidney must be due to the capacity of the latter tissue to desaminate the cytidylic acid moiety. It would seem probable that extracts of rat kidney and spleen desaminate only the adenylic acid and guanylic acid moieties while those of mouse kidney desaminate the moieties of all three aminated nucleotides. Mouse-kidney extracts apparently effect close to the maximum desamination and dephosphorylation of ribosenucleate (Fig. 3). The relatively close concordance of the Fiske-Subbarow and Lowry-Lopez methods of phosphate estimation suggests that the greater part at least of the inorganic phosphate measured in the digests with nucleates actually represents phosphate split from the substrates enzymatically and not a product that is the result of hydrolysis of acid-labile phosphate esters by the reagents used in the former determination.

The Appearance of Dialyzable Components during the Digestion of the Nucleates with Tissue Extracts

Only a very small fraction of the nucleate solutions by themselves, of the tissue extracts by themselves, or of mixtures of boiled extracts with nucleates, is dialyzable through a cellophane membrane. However, during the incubation of fresh tissue extracts with nucleates, an appreciable proportion of nucleate material diffuses through the membrane. Thus, simultaneously with the appearance of ammonia and of inorganic phosphate in the digests of the nucleates there occurs a degradation of the molecules to particles small enough to pass through a cellophane membrane.

Table 7 is a summary of typical experiments

with tissue extracts prepared from livers and spleens of freshly killed rats. The procedure was as follows: The organs were removed, ground with sand, and extracted with three volumes of water, followed by light centrifugation. Two cubic centimeters of spleen extract or four cubic centimeters of liver extract was added to 10 mg. of nucleate in solution of distilled water to make a total volume inside the dialysis bag of 5 cc. The outside volume was 20 cc. Under these conditions, it was found necessary to add salts to the system to obtain optimal degradation of desoxy-

periods of incubation of yeast nucleic acid and thymus nucleic acid with aqueous extracts of liver.

Liver from freshly killed rats was extracted with three volumes of water; 4 cc. of the extract was added to 2 cc. of a 0.5% solution of nucleates within a cellophane bag. Sodium chloride was added outside the bag to a concentration of 0.01 N. The volume outside the bag was 20 cc. The data in Table 8 were calculated on the basis of the total volume (26 cc.).

The formation of ammonia did not precede the

TABLE 7. CONCENTRATION OF DIALYZABLE COMPONENTS FROM NUCLEIC ACID DIGESTS

Tissue extract	Nucleate[1]	Dialyzable products formed[2]						
		Total N	Ammonia N	Purine N	Total P	Xanthine	Ribose	Desoxy-ribose
		Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
Spleen	Ribosenucleic acid	1.04	0.16	0.314	0.90	0.53	1.5	—
	Desoxyribosenucleic acid	1.12	.17	.291	.93	.46	—	2.3
Liver:								
Experiment 1	Ribosenucleic acid	1.14	.10	.285	.73	.60	2.4	—
	Desoxyribosenucleic acid	1.14	.12	.303	.65	.56	—	2.6
Experiment 2	Ribosenucleic acid	1.25	.12	—	.92	.58	2.82	—
	Desoxyribosenucleic acid	1.17	.12	—	.92	.60	—	2.15
Experiment 3	Ribosenucleic acid	1.12	.10	—	.60	—	2.6	—
	Desoxyribosenucleic acid	1.12	.10	—	.59	—	—	1.9

[1] 10 mg. of nucleate used for each digest.

[2] Data were calculated on the basis of total volume of system (25 cc.). In theory, 10 mg. of nucleic acid should yield 1.5 mg. nitrogen, 0.9 mg. phosphorus, 4.3–4.6 mg. pentose, 0.3 mg. ammonia, and approximately 0.85 mg. desaminated purines.

ribosenucleic acid. Sodium and magnesium salts were compared and found equally effective over a concentration range of 0.001 M to 0.006 M. Ribosenucleic acid metabolism was apparently independent of salt concentration under the conditions of these experiments. However, these findings hold only for the conditions given. In more dilute systems, the nature and concentration of the salts profoundly influence the metabolism of both ribosenucleic and desoxyribosenucleic acids.

These data show that with the appearance of ammonia in digests of nucleic acids with tissue extracts, there are formed dialyzable nitrogen and phosphorus compounds and equivalent amounts of pentose. It is also apparent that of the total amino nitrogen available (300 micrograms per 10 mg. of nucleate) only about two-thirds appears as ammonia nitrogen. This finding is in agreement with those of Greenstein and Chalkley (1945) and is a consequence of the inability of rat tissues to desaminate cytidylic acid (Greenstein, Carter, Chalkley and Leuthardt, 1946).

In Table 8 the concentration of various components in the dialyzate are recorded for increasing

appearance of other products and increased in approximately constant proportion to nitrogen and phosphorus on continued incubation. During early periods of incubation, thymus nucleate was enzymatically degraded to an extent considerably less than that of yeast nucleate, although on longer incubation practically identical values for dialyzable nitrogen and phosphorus were obtained.

The effect of increasing concentrations of tissue extract (rat liver) upon constant quantities of nucleates is shown in Table 9. In this experiment, livers from freshly killed rats were extracted with three volumes of water and added to 2 cc. of a 0.5% solution of nucleates in cellophane bags as indicated in the first column of Table 9. The volume outside the bag was 20 cc.; the data were calculated on the basis of total volume. Sodium chloride was added to the outside volume to a concentration of 0.01 N. The incubation period was 4 hours.

Again the difference in metabolism of ribosenucleic acid and desoxyribosenucleic acid is seen at low concentrations of tissue extract, but as the quantity of extract increased, this difference was overcome. Although increasing concentrations of extract pro-

duced increases in total dialyzable nitrogen, there was at no point complete desamination of total available amino groups (300 micrograms of ammonia per 10 mg. nucleate). The ratio of ammonia nitrogen to total dialyzable nitrogen was greater at lower extract concentrations.

Spectrophotometric Data

Purines and pyrimidines are characterized by ultraviolet absorption spectra exhibiting well-defined maxima in the region 2400 A to 2900 A. Ultraviolet microscopy has been extensively used as an instrument for the quantitation of nucleic acids in cells, based on the characteristic maxima of these compounds at 2600 A. It was found that the transmission of ultraviolet light through dialyzates of nucleic acid digests exhibited the characteristic absorption of intact nucleic acid. Incubation of nucleic acid with tissue extracts under the conditions employed, aside from slight shifts in the 2600 A maxima to longer wavelength (2650 A-2690 A), produced no alteration in the purine or pyrimidine components that was reflected in the absorption spectra. An exception to this finding was noted in nucleate digests with rat-hepatoma extracts described later.

Figure 8 is a comparison of the absorption spectra of a composite of nucleotides equivalent in concentration to the proportion of adenylic, guanylic, uridylic, and cytidylic acid found in yeast nucleic acid, and the absorption spectra of an equivalent concentration of yeast nucleic acid based on total phosphorus content.

Similar data for thymus nucleic acid were published by Loofbourow and Stimson (1940). From the foregoing, it is apparent that degradation of nucleic acid into nucleotides would not be expected to produce alterations in the absorption spectra. It was shown by Kalckar (1944, 1945) that desamination of adenylic acid produces a slight shift of absorption maxima to shorter wavelengths and that more profound alterations of the absorption spectra

Table 8. Relation of Incubation Period to Concentration of Dialyzable Products in Digests[1] of Nucleates with Rat-liver Extract

Incubation period (in hours)	Nucleate	Dialyzable products				
		Total N	Ammonia N	Ribose	Desoxyribose	Total P
		Mg.	Mg.	Mg.	Mg.	Mg.
2	Ribosenucleic acid	0.44	0.03	Trace	—	0.20
	Desoxyribosenucleic acid	.01	.003	—	0.13	.02
4	Ribosenucleic acid	.53	.045	0.89	—	.23
	Desoxyribosenucleic acid	.53	.045	—	1.04	.23
6	Ribosenucleic acid	.88	.10	1.69	—	.48
	Desoxyribosenucleic acid	.88	.10	—	1.80	.50

[1] 10 mg. ribosenucleic acid or desoxyribosenucleic acid used in each digest.

Table 9. Effect of Concentration of Tissue Extract on Formation of Dialyzable Products

Volume of extract (in cubic centimeters)	Nucleate[1]	Dialyzable products formed					
		Total N	Ammonia N	Total P	Inorganic P	Ribose	Desoxyribose
		Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
1	Ribosenucleic acid	0.62	0.08	0.37	0.13	0.82	—
	Desoxyribosenucleic acid	.14	.04	.12	.06	—	1.35
2	Ribosenucleic acid	.65	.06	.41	.19	1.8	—
	Desoxyribosenucleic acid	1.00	.05	.32	.19	—	1.63
3	Ribosenucleic acid	.99	.10	.45	.16	1.8	—
	Desoxyribosenucleic acid	.99	.09	.45	.16	—	1.82
4	Ribosenucleic acid	1.25	.11	.92	.35	1.8	—
	Desoxyribosenucleic acid	1.17	.12	.92	.35	—	2.15

[1] 10 mg. of ribosenucleic acid or of desoxyribosenucleic acid in digest adjusted to constant volume 6 cc. by addition of water.

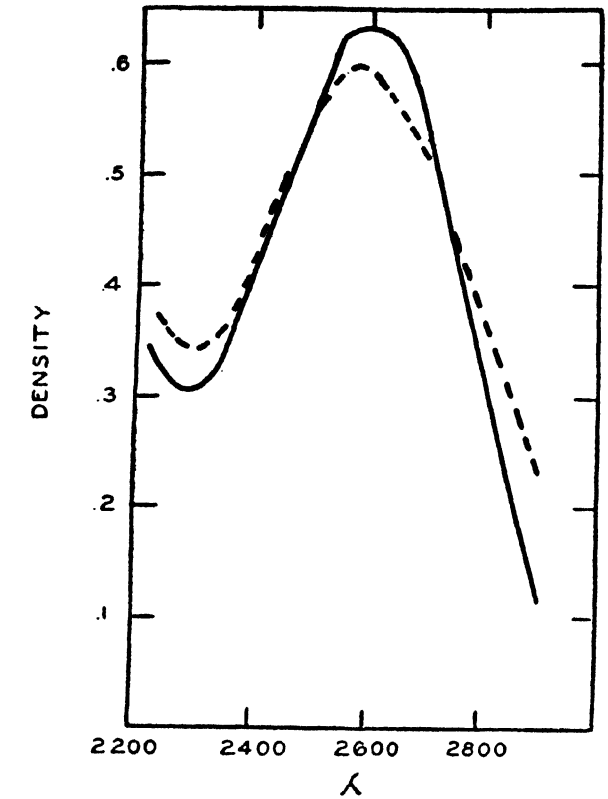

FIG. 8. Absorption expressed in terms of density scale readings on the Beckman spectrophotometer. Solid line represents absorption of ribosenucleic acid and dotted line absorption of an equimolar mixture of guanylic, adenylic, uridylic, and cytidylic acids in equivalent concentration based on total phosphorus content equal to that of the nucleate.

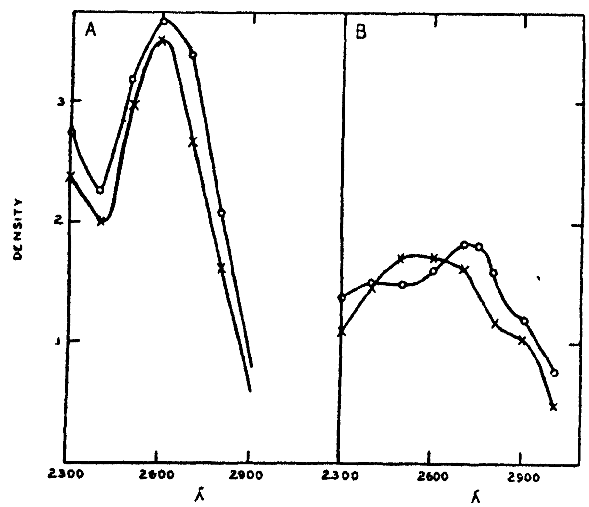

FIG. 9. *A*, Normal rat liver; *B*, Rat hepatoma 31. Crosses on figure designate ultraviolet absorption in dialyzates of digests containing ribosenucleic acid; circles represent similar data for desoxyribosenucleic acid. Curves were corrected for blanks on tissue extract in absence of nucleate.

accompany xanthine oxidase activity. Apparently the substances in the dialyzates are not substrates for xanthine oxidase (i.e., nucleosides), or the enzyme is not active under the conditions employed.

It is then possible to use the absorption of light at 2600 A in the dialyzates as a measure of total dialyzable components. The latter term is used with no commitment as to the nature of the linkage involved.

The use of ultraviolet spectrophotometric measurements considerably facilitates study of rates of appearance of dialyzable components. It is imperative, however, in applying this method that the absorption pattern for the components in the digest dialyzate coincides with that of the intact nucleate; otherwise, basing quantitative measurement on the extent of absorption at 2600 A will be misleading when applied to solutions containing oxidation products of purines and pyrimidines in which the absorption pattern is altered (lower maxima at longer wavelengths). To illustrate this point in Figure 9 are contrasted the ultraviolet absorption spectra of dialyzates of ribosenucleate and desoxyribosenucleate which had been incubated with normal rat liver, with dialyzates of similar digests with hepatoma 31. In this experiment normal rat liver and rat hepatoma 31 were ground with sand and extracted with 30 volumes of water, followed by light centrifugation. Three cubic centimeters of each of these extracts was then added to solutions containing 0.5 mg. of ribosenucleic and desoxyribosenucleic acid in cellophane bags as previously described, and increments in absorption at 2300 A to 3000 A in the dialyzates were measured after 4 hours of incubation. Blanks contained distilled water in place of nucleates. Sodium chloride was added to a concentration of 0.01 N in all tubes.

The differences in absorption spectra are attributed to the increased oxidation of the purine and pyrimidine components in hepatoma digests as compared with normal liver.[1] Greenstein and Chalkley (1945) showed that extracts of hepatomas produce more ammonia on incubation with ribosenucleic and desoxyribosenucleic acids than do extracts of normal liver. Kalckar (1945) found that oxidation of purines by xanthine oxidase causes a decrease in absorption at 2600 A with compensatory increase at 2900 A. In view of these findings, the Caspersson data (Caspersson and Santesson, 1942) relating nucleic acid concentration to protein synthesis based on the appearance of maxima around 2800 A are open to the interpretation that such findings represent oxidation products of purines and not necessarily increased concentrations of certain amino acids.

Results of the application of spectrophotometric determination of total dialyzable components from digests of nucleates with rat tissue extracts are recorded in Table 10. Specific extinction coefficients

[1] This finding was not constant in all dialyzates of hepatoma digests.

have not been used because of the lack of adequate characterization of the substrate and because of the facility with which density scale readings on the spectrophotometer may be expressed as percent dialyzable components. Thus a solution containing 0.5 mg. nucleate in 25 cc. water gives a density reading on the spectrophotometer of about 0.460, and the dialyzates of nucleate digests which originally contained 0.5 mg. of nucleate within the cellophane bag, having an absorption at 2600 A of 0.230, would contain dialyzable components accounting for about 50% of the added nucleate. Less than 5% of either nucleate was spontaneously dialyzable within the period of incubation.

The experimental conditions under which the data of Table 10 were obtained were the following: Rat tissues were ground with sand and extracted with three volumes of water followed by light centrifugation; digests consisted of 1 cc. of extract

TABLE 10. FORMATION OF DIALYZABLE COMPONENTS FROM NUCLEATE DIGEST WITH TISSUE EXTRACT

Rat-tissue extracts	Dialyzable components of nucleates formed in digests[1]	
	Ribosenucleic acid	Desoxyribose-nucleic acid
	Per cent	*Per cent*
Spleen	50	44
Liver	60	30
Kidney	70	50
Pancreas	60	20
Brain	40	10
Muscle	30	2
Dialyzed Spleen	55	3

[1] 4-hour incubation period.

and 1 cc. of a 0.5% solution of nucleate made to a volume of 5 cc. within the cellophane bag of the dialysis set-up previously described. The outside volume was 20 cc., and sodium chloride was added to a concentration of 0.01 N. Dialyzates were diluted 1:10 with distilled water before reading.

These data show considerable variation in the ability of the various tissues to degrade desoxyribosenucleic acid into dialyzable components, muscle being virtually inactive while kidney and spleen produce about 50% dialyzable components in a 4-hour incubation period under these conditions. Ribosenucleic acid is more susceptible to degradation, and there is less variation in the metabolism of this material by different tissues. By dilution of tissue extracts it was possible to eliminate the capacity to degrade desoxyribosenucleic acid at extract concentrations which retained considerable activity toward ribosenucleic acid. Dialysis caused tissue extracts to lose their ability to degrade desoxyribosenucleic acid to dialyzable components, and the addition of salts (with the exception of bicarbonate) to such extracts completely restored this activity.

EXPERIMENTS WITH CRYSTALLINE RIBONUCLEASE

In digests containing high concentration of tissue extracts and nucleates electrolytes were found to have no appreciable effect on the metabolism of ribosenucleic acid, while they accelerated the breakdown of desoxyribosenucleic acid. Under conditions employing concentrations of nucleate and extract about one-fifth to one-tenth that of experiments previously described, it was possible to show that the presence of electrolytes markedly accelerated the degradation of ribosenucleic acid into dialyzable components and that the greater part of this could be accounted for by the effect of the salt in the absence of any enzyme. Figure 10 illustrates the effect of sodium chloride on the formation of dialyzable components from ribosenucleic acid in the presence and absence of crystalline ribonuclease (Kunitz, 1940) (Kunitz preparation) as compared

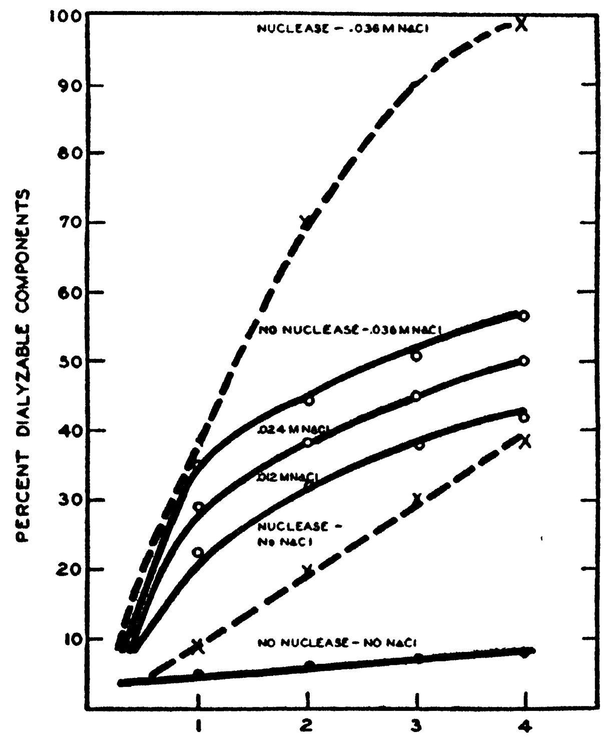

FIG. 10. Influence of electrolyte upon formation of dialyzable components from ribosenucleic acid in the presence and absence of crystalline ribonuclease. Cellophane bag contained 1 mg. of ribonucleate and 0.1 mg. of crystalline ribonuclease in total volume of 3 cc. Salt concentration in system varied as shown in graph. The lowermost line represents the spontaneous break-down of ribosenucleic acid in distilled water. The lower broken line shows the formation of dialyzable components from the nucleate in the presence of ribonuclease, and the upper broken line the effect of nuclease and salt. The intermediate solid lines represent the desaggregating effect of salt alone.

with the spontaneous break-down of the compound.

These data show that there is a slight, progressive, spontaneous break-down of ribosenucleic acid to dialyzable components, which is accelerated many times by the presence of salts. Magnesium ions have been found to be about four times as effective as sodium ions in this regard. The effect is proportional to concentration and is characterized by a rapid initial appearance of dialyzable components followed by a progressive but slower rate of degradation. In Fig. 10 the effect of crystalline ribonuclease upon the degradation of ribosenucleic acid in the

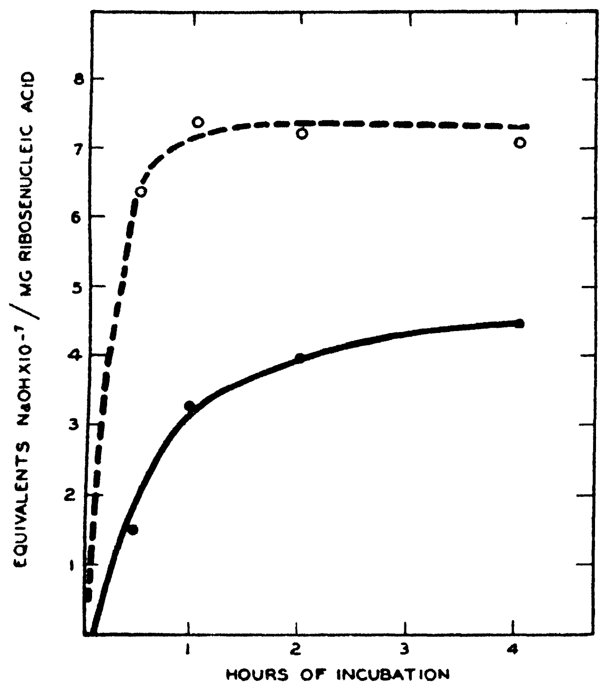

FIG. 11. Influence of electrolyte upon the liberation of acid groups from ribosenucleic acid by crystalline ribonuclease. Broken line indicates nuclease activity in presence of Mg^{++}; solid line, in absence of salt.

presence and absence of salt is illustrated; from the slope of the lines representing the appearance of dialyzable components it is apparent that salts accelerate enzymatic activity. Therefore, the appearance of dialyzable components from digests of ribosenucleic acid is not *per se* a measurement of enzymatic activity, for the same effect is accomplished nonenzymatically by electrolytes. Because of the heat-stable nature of the ribonuclease, it is difficult to make an easy distinction between enzymatic and nonenzymatic activity in tissue extracts. However, the nature of the effect produced by electrolytes and that produced by crystalline ribonuclease can be easily differentiated by the ability of the latter to liberate acid groups in the course of the degradation of ribosenucleic acid. Ten milligrams of ribosenucleic acid and one milligram of crystalline ribonuclease in a total volume of 10 cc. were incubated at 39° for 4 hours, in the presence and absence of 0.1 M electrolyte, and at the end of the period were titrated with 0.001 N sodium hydroxide to first change of color of phenolphthalein. Controls consisted of the same amount of nucleic acid in water and in salt solutions. Data expressed as increments of titratable H^+ per milligram of nucleate over ribosenucleic acid blanks revealed that ribonuclease alone liberated 4.56×10^{-7} equivalents H^+; ribonuclease plus sodium chloride 6.5×10^{-7} equivalent H^+; and ribonuclease plus magnesium chloride, 7.00×10^{-7} equivalents H^+; while sodium chloride and magnesium chloride in the presence of ribosenucleic acid did not liberate titratable acid groups. It is significant that although salts are capable of causing the degradation of ribosenucleic acid to dialyzable components, no acid groups are liberated in the process, suggesting that the process is one of desaggregation, as contrasted with the splitting of the doubly esterified phosphate bonds, which characterize the activity of the crystalline ribonuclease. The liberation of acid groups by the crystalline enzyme is nevertheless influenced to a considerable extent by salt concentration as illustrated in Fig. 11. These data show that in the absence of salt the enzyme liberates 0.65 acid equivalents per mole of ribosenucleic acid (molecular weight of 1,286) and in the presence of 0.1 M magnesium chloride, 0.94 acid equivalents per mole. In Figure 11 the influence of magnesium (0.1 M magnesium chloride) upon the rate of liberation of acid groups in digests of ribosenucleic acid with crystalline ribonuclease is shown. The acceleration of the rate of reaction is striking in the presence of magnesium ions. This has been shown to be proportional to the conceneration of the electrolyte, and to be greater in the presence of the divalent magnesium ion than in solutions of equal normality of the monovalent sodium ion.

EXPERIMENTS WITH PURIFIED DESOXYRIBONUCLEASE

A preparation of purified desoxyribonuclease from beef pancreas was recently described by McCarty (1946). Because of the dependence of this enzyme upon salt activation (Mg^{++}, Mn^{++}), data relating the behavior of these enzymes to the phenomena associated with the metabolism of nucleic acids by dialyzed tissues were desired. For this purpose a sample of purified desoxyribonuclease was obtained through the kindness of Dr. Maclyn McCarty, of the Rockefeller Institute.

McCarty's measurements of desoxyribonuclease activity were based on viscosimetric data. The enzyme was activated only by Mg^{++} and Mn^{++} and the Mg^{++} activation was destroyed by fluoride. Ribonuclease was shown by Kunitz (1940) to form dialyzable products when incubated with ribose-

nucleic acid. Similar data were not available for desoxyribonuclease activity. A series of experiments was therefore made utilizing the systems previously described for detection by spectrophotometric measurement of dialyzable components resulting from nucleic acid digests.

Dialysis Data

In addition to the spectrophotometric assay for dialyzable components, an estimate of acid precipitability was made upon the contents of the cellophane bags (desoxyribosenucleic acid), by comparing the turbidity produced upon addition of dilute hydrochloric acid.

Cellophane bags were inserted into centrifuge tubes containing 20 cc. of distilled water brought to the desired salt normality; and to the bags were added 1 cc. of desoxyribonuclease solution containing 0.1 mg. of purified enzyme, 1 cc. of a 0.1% solution of desoxyribosenucleate (previously dialyzed to remove traces of sodium chloride), and 3 cc. of distilled water.

TABLE 11. ACTIVATION OF PURIFIED DESOXYRIBONUCLEASE BY SALT

Purified enzyme (in milligrams)	Desoxyribosenucleic acid	Salt	Acid precipitability of substrate following incubation at—		Absorption at 2600 A in dialyzate (density)
			1 hour	3 hours	
0	1.0	O	100	100	0.025
0.1	1.0	O	90	44	.025
0.1	1.0	LiCl	42	0	.030
0.1	1.0	NaCl	56	20	.050
0.1	1.0	KCl	56	0	.040
0.1	1.0	RbCl	28	20	.050
0.1	1.0	CsCl	52	0	.060
0.1	1.0	NH_4Cl	66	30	.040
0.1	1.0	$CaCl_2$	0	0	.185
0.1	1.0	$MgCl_2$	0	0	.225
0.1	1.0	$SrCl_2$	0	0	.170
0.1	1.0	$BaCl_2$	26	0	.225
0.1	1.0	Guanidine HCl	56	20	.065
0.1	1.0	Arginine HCl	0	0	.100
0.1	1.0	Na glutamate	20	0	.095
0.1	1.0	$MnCl_2$	0	0	.325
0.1	1.0	Zinc acetate	54	54	.027
0.1	1.0	$NaHCO_3$	100	100	.025
0.1	1.0	Na_2HPO_4	54	70	.040
0.1	1.0	NaF	30	10	.045
0.1	1.0	Sodium citrate	97	100	.030
0.1	1.0	Albumin (egg)	0	0	.115
0.1	1.0	Glycyl glycine	95	100	.025

Acid precipitability was determined upon the contents of the bag by transferring to test tubes and adding 0.2 cc. of 0.1 N hydrochloric acid. The turbidity produced was compared with a standard composed of 1 mg. of desoxyribosenucleic acid in 5 cc. of water similarly treated. Turbidity was compared in a visual colorimeter with the standard set at 20, and acid precipitability was reported as reciprocals of test readings multiplied by 2×10^4. Thus, the standard is 100, and decreases in acid precipitability give decreasing values. This is, of course, a rough estimate of the reaction of desoxyribosenucleic acid and is intended only as an expression of the altered turbidity of nucleate-enzyme digests following incubation in the presence of various salts.

Absorption at 2600 A was measured, in the dialyzates, of the various solutions with the Beckman spectrophotometer. As previously mentioned, this was found to be an index of the dialyzable nucleic acid, or total dialyzable components.

Table 11 gives the results of experiments in which a wide variety of salts at equal normality was investigated as activators for desoxyribonuclease.

From the data (Table 11), it is evident that a variety of monovalent and divalent ions and three organic compounds (arginine hydrochloride, sodium glutamate, and purified egg albumin (sodium albuminate)) are capable of activating the enzyme sufficiently to alter acid precipitability. These changes are obvious upon addition of acid, and one need not rely upon determination of turbidity for detection of change. In this regard, we noted that solutions containing higher concentration of enzyme to substrate (0.4 mg. enzyme to 0.1 of desoxyribosenucleic acid) showed almost complete loss of acid precipitability of substrate upon incubation in the absence of ion activation. Under these conditions there was no formation of dialyzable products.

Although a wide variety of ions was capable of activitating the purified enzyme, there was considerable difference within the group in accelerating changes in acid precipitability of desoxyribosenucleic acid and particularly in the formation of dialyzable components, the divalent ions being more efficient in both cases. Loss of acid precipitability determined by these techniques apparently precedes and may be complete in the absence of the formation of dialyzable components. The organic radicals occupied a position of activating efficiency somewhat greater than the monovalent metallic ions tested. It is noteworthy that sodium fluoride had an activating effect and that sodium bicarbonate provided no activation.

Figure 12 shows the rates of formation of dialyzable components from systems activated by various salts. The experimental procedure was as follows: To the cellophane bag were added 1 cc. of purified nuclease solution containing 0.1 mg. of purified enzyme, 1 cc. of 0.1% desoxyribosenucleic acid, and 3 cc. of water. To the outside volume (20 cc.) salt was added to a concentration of 0.01 N. Absorption at 2600 A in dialyzate was measured by means of the Beckman spectrophotometer and

was reported in terms of density readings. For this comparison, the interval between the first and second hours of incubation was selected, and the rates were calculated from the slope of the line. The high activating power of divalent ions is apparent.

The absorption spectra of the dialyzates of these systems showed characteristics identical with those of intact desoxyribosenucleic acid. Under the conditions of the experiment, about 70% of the desoxyribosenucleic acid was degraded into dialyzable components. Neither ammonia nor inorganic phosphate was liberated.

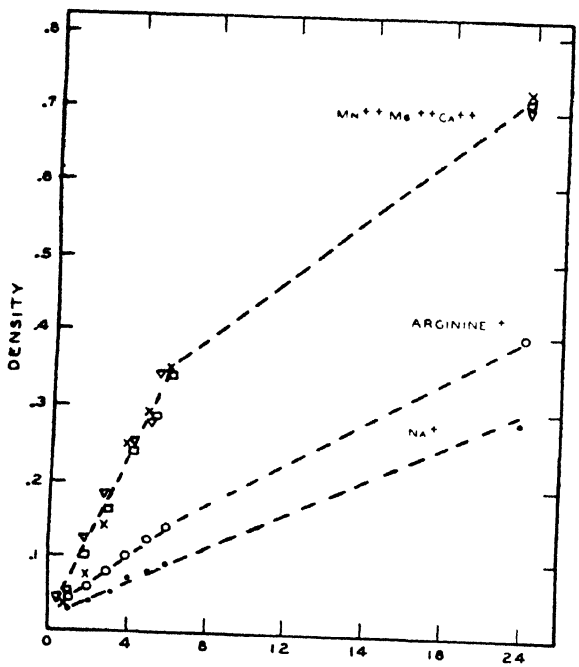

FIG. 12. Effect of salts on formation of dialyzable components from desoxyribosenucleate in the presence of purified nuclease. All salts were at 0.01 N concentration.

The comparative activating effects of sodium and magnesium ions as a function of concentration are shown in Fig. 13. In this case, the solution within the cellophane bag contained 0.1 mg. purified nuclease and 1 mg. desoxyribosenucleic acid. Salts were added to the systems in the concentrations shown in the graph. The incubation period was 3 hours. The absorption in the dialyzate was measured at 2600 A and was recorded in density readings (or percent dialyzable components $\times 10^5$). Both ions exhibited maximal effective concentrations, above which this activating power diminished. Magnesium ions were several hundred percent more effective in accelerating the formation of dialyzable components than were sodium ions in similar concentrations. It is noteworthy that sodium ions exhibited maximal activity in physiologic concentration.

Bicarbonate ions proved inhibitory when added to Mg^{++}-activated system in concentrations of 10 to 30 millimolar. This effect was found to be independent of the shift to alkaline pH ranges in the unbuffered systems, for addition of sodium hydroxide in amounts sufficient to produce comparable pH changes resulted in little or no inhibition.

The addition of fluoride to Mg^{++}-activated systems resulted in no inhibition of formation of dialyzable components in the concentrations used (0.01 N to 0.03 N sodium fluoride).

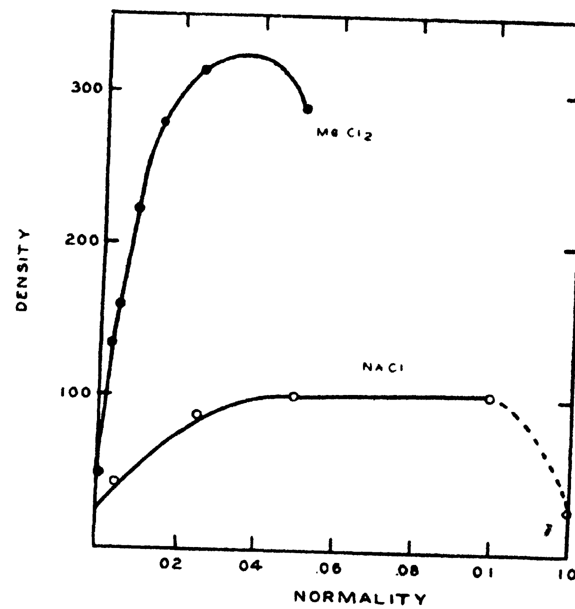

FIG. 13. Effect of varying concentrations of magnesium and sodium chlorides on formation of dialyzable components from desoxyribosenucleate in the presence of purified nuclease.

As in the case of ribonuclease acting upon ribosenucleic acid (Kunitz, 1940), purified desoxyribonuclease was found to produce a shift to acid pH when incubated with solutions of desoxyribosenucleic acid, without producing inorganic phosphate.

VISCOSIMETRIC DATA

Profound alterations of viscosity are induced in solutions of desoxyribosenucleic acid upon enzymatic degradation of this highly polymerized substance. The viscosimetric techniques employed varied from the technique described by McCarty (1946) in that higher concentrations of desoxyribosenucleic acid were used (final concentration, 0.5%) and measurements were carried out under a constant external pressure gradient. Under these conditions viscosity is a function of molecular asymmetry. From data thus collected, it was found that Mg^{++}, Mn^{++}, arginine hydrochloride, sodium gluta-

mate, and sodium albuminate (purified egg albumin) had high activating effects while Ca^{++}, Na^{+}, NH_4^{+}, K^{+}, Ba^{++}, Zn^{++}, Fe^{++}, F^{-}, Cs^{+}, Rb^{+} and Li^{+} had low but detectable activating effects. At equimolar or slightly higher concentrations, sodium fluoride did not inhibit the Mg^{++}-activated enzyme. Although bicarbonate inhibited formation of dialyzable components from Mg^{++}-activated systems, as previously described, no inhibiting effect could be demonstrated on Mg^{++}-activated systems studied by viscosimetric techniques.

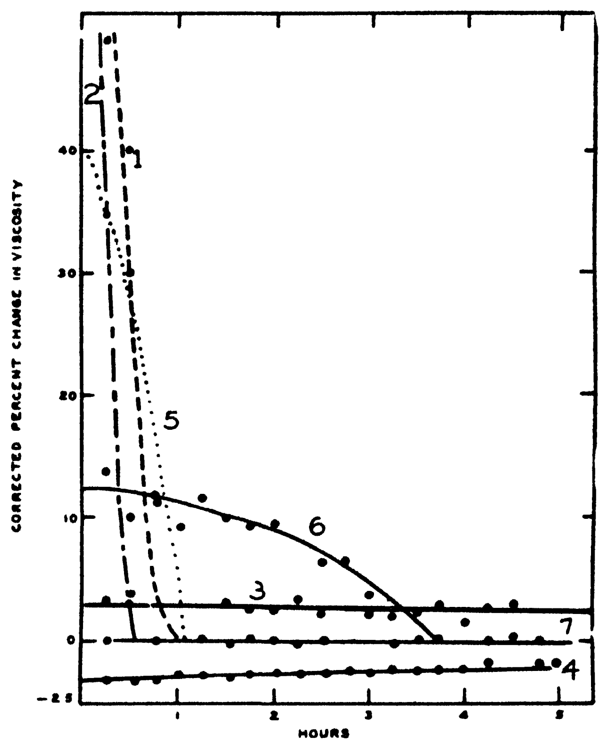

FIG. 14. Percent change in viscosity at constant external pressure of 16 cm. water and at 30° C of mixture of 3 cc. dialyzed 1% sodium desoxyribosenucleate, 1 cc. 0.01% purified desoxyribonuclease, 1 cc. of salt solution, and 1 cc. water; 5 cc. of the mixtures was incubated in the viscometer. Results given in ordinate in terms of percent change in viscosity at each time interval from which was subtracted the percent change at the same interval of a similar mixture in which 1 cc. of water was present in place of 1 cc. of salt. Abscissa, period of incubation. Curve 1 describes results in the presence of: 1 cc. of 0.15 N magnesium chloride alone, or 1 cc. of a mixture of 0.15 N magnesium chloride and 0.15 N sodium fluoride, or 1 cc. of a mixture of 0.15 N magnesium chloride and 0.23 N sodium fluoride, or 1 cc. of a mixture of 0.15 N magnesium chloride and 0.23 N sodium bicarbonate. Curve 2 refers to results in the presence of 1 cc. 0.15 N manganese chloride, curve 3 to results in the presence of either 1 cc. 0.15 N sodium chloride or 1 cc. 0.15 N sodium fluoride, curve 4 to results in the presence of 1 cc. 0.15 N sodium bicarbonate, curve 5 to results in presence of 1 cc. 0.15 N sodium- *l*-glutamate, curve 6 to results in the presence of 1 cc. 0.15 N *l*-arginine monohydrochloride, and curve 7 to results in the presence of 1 cc. 0.15 N calcium chloride. In all cases the solution of purified nuclease was added to the mixture last.

Figure 14 illustrates the activating effects of several ions and organic compounds assayed by viscosimetric methods, and in the following tabulation acid-soluble phosphorus present in mixtures of desoxyribosenucleic acid, purified desoxyribonuclease, and salts is recorded. The digest mixtures were the same as those described in Fig. 14 and were removed from the viscometers at the end of the 5-hour period of incubation. After adding 0.1 cc. of 1 N hydrochloric acid to 2.5 cc. of the digest, the precipitate was filtered off and the filtrate analyzed for total phosphorus. Results are given in terms of 5 cc. of digest containing 2,250 micrograms of nucleic acid phosphorus. The manganous ion was the most potent activator. Sodium fluoride and sodium chloride provided slight but equal activation, while sodium glutamate and arginine monohydrochloride occupied intermediate positions. In the magnesium-activated systems, high concentrations of sodium fluoride (0.23 N) did not inhibit fall in viscosity or formation of acid-soluble phosphorus.

Salt[1]	Acid-soluble phosphorus Micrograms
No salt	0
0.15 N Manganese chloride	240
0.15 N Magnesium chloride	228
0.15 N Magnesium chloride +0.15 N sodium fluoride	220
0.15 N Magnesium chloride +0.23 N sodium fluoride	220
0.15 N Sodium glutamate	180
0.15 N Arginine monohydrochloride	40
0.15 N Sodium chloride	20
0.15 N Sodium fluoride	20
0.15 N Sodium bicarbonate	0
0.15 N Calcium chloride	0

[1] 1 cc. of salt of designated normality in 6 cc. of the digestion mixture.

In studies of formation of dialyzable components from desoxyribosenucleic acid in the presence of purified desoxyribonuclease, calcium was found to be strongly activating. Viscosimetric data, on the other hand, showed no calcium activation, in agreement with McCarty (1946). However, it should be noted that the ratio of concentration of salt to concentration of substrate in systems for viscosity studies was much lower than that in dialysis studies, while the same concentration of purified enzyme was used throughout. Another point of interest was the ability of purified egg albumin (sodium albuminate, pH 6.9) to activate the purified desoxyribonuclease.

TITRATION DATA

The liberation of acid groups during the enzymatic degradation of nucleic acids is a consequence of the splitting of the double esterified phosphate linkages between nucleotides and is probably the best criterion of this phase of the enzymatic degradation process. As shown in this review, ribosenucleic acid may be so altered in the presence of electro-

lytes as to become dialyzable while failing to liberate acid groups in the process. In contrast, the crystalline enzyme ribonuclease besides degrading the nucleic acid into dialyzable components also liberates titrable acid groups, processes which are accelerated by salts.

In order to assess adequately the role of electrolytes in the activation of purified desoxyribonuclease, it was necessary to determine the ability of the enzyme to liberate acid groups alone and in the presence of salts. The results of titration experiments, conducted as described for ribosenucleate, in which 10 mg. of desoxyribosenucleic acid and 1 mg. of purified desoxyribonuclease in a total volume of 10 cc. were incubated at 37° for 4 hours in solutions of distilled water, and either 0.07 M magnesium chloride or 0.1 M sodium chloride, were expressed as increments of titratable H^+ per milligram of nucleate over desoxyribosenucleic acid blank. Desoxyribonuclease alone liberated 1.1×10^{-7} equivalents H^+; desoxyribonuclease plus sodium chloride, 3.2×10^{-7} equivalents H^+; and desoxyribonuclease plus magnesium chloride, 5.4×10^{-7} equivalents of H^+. Controls consisting of the nucleate in solution of water and the two salts showed that as in the case of ribosenucleic acid electrolytes alone were incapable of liberating acid groups. However, unlike the case of ribosenucleic acid, salts alone at these concentrations were incapable of forming dialyzable components from solution of desoxyribosenucleate.

The results of these experiments are in agreement with those obtained by viscosimetric and dialysis techniques, namely, that the purified enzyme alone, although possessing slight activity in degrading desoxyribosenucleic acid, is accelerated many times by the presence of electrolyte and that the divalent magnesium ion is more effective than the monovalent sodium ion, at nearly equivalent concentrations.

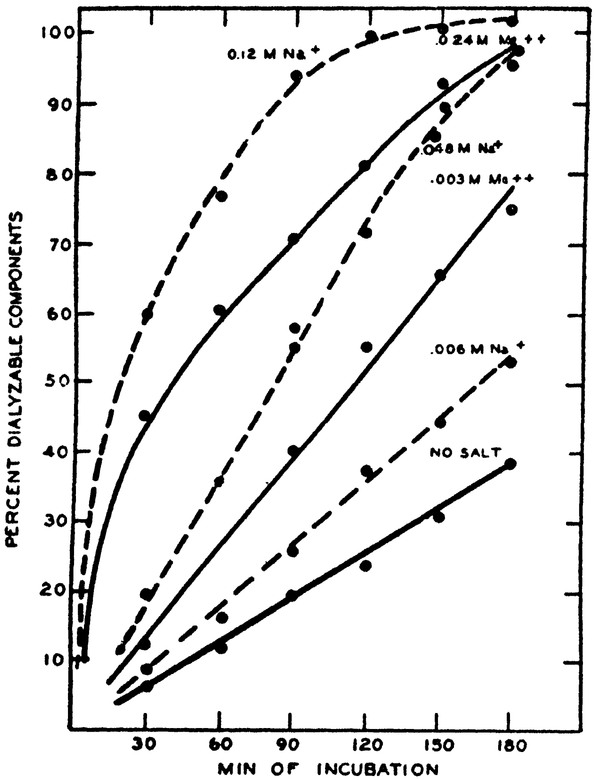

FIG. 15. Formation of dialyzable components from a digest of rat-kidney extract and ribosenucleic acid and the effect of electrolytes.

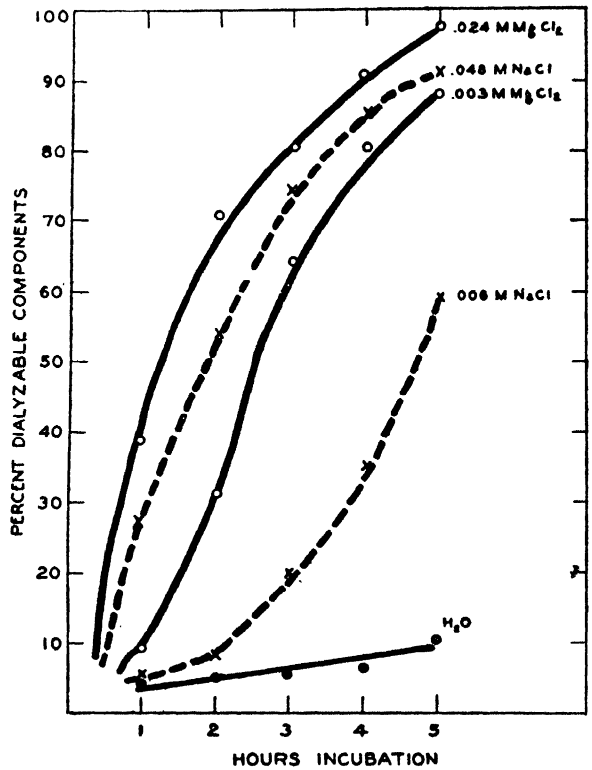

FIG. 16. Formation of dialyzable components from a digest of rat-kidney extract and desoxyribosenucleic acid and the effect of electrolytes.

Experiments with Tissue Extracts

In general the results of experiments with the purified enzymes can be applied to nucleate digests with tissue extracts. It has been shown (Carter and Greenstein, 1946a) that dialysis removes the capacity of tissue extracts to desaminate and dephosphorlyate desoxyribosenucleic acid, and that this capacity can be restored by the addition of many kinds of electrolytes. Experiments with the purified desoxyribonuclease have demonstrated that the electrolyte phenomenon is concerned with the enzymatic

degradation of the intact nucleate to smaller fragments with the liberation of acid groups. A similar influence of electrolytes upon ribosenucleic acid degradation can be shown, but because of the smaller particle size of the polymerized ribosenucleic acid the effect is of a different order. Figures 15 and 16 illustrate the influence of salts upon the degradation of nucleic acids to dialyzable components by an extract of rat-kidney. In these experiments rat kidney was ground with sand and extracted with 30 volumes of water followed by light centrifugation. For the degradation of ribosenucleic acid, 1 cc. of this extract was added to 1 cc. of a 0.1% solution of ribosenucleic acid within cellophane bags in the dialysis set-up previously described. Experiments with desoxyribosenucleic acid were conducted with 2 cc. of the kidney extract and 1 mg. of desoxyribosenucleic acid (1 cc. of a 0.1% solution).

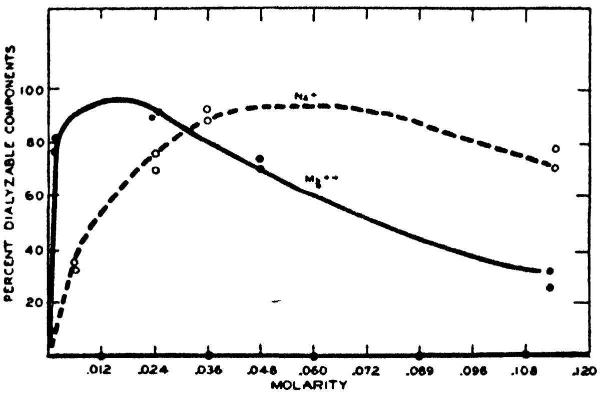

FIG. 17. Effect of concentration of electrolyte upon the formation of dialyzable components from digests of desoxyribosenucleic acid.

As shown previously in our experiments with isolated systems, the influence of salts upon the enzymatic degradation of ribosenucleic acid cannot be adequately assessed by employing criteria of formation of dialyzable components, for the electrolytes themselves produce a desaggregation of the particles with progressive liberation of dialyzable components. Furthermore, this effect is related to the concentration of salt employed and the valency of the ion. These data, however, show an acceleration of the degradation of ribosenucleic acid by tissue extracts in the presence of salts; but by the nature of the phenomena it is difficult to determine how much of the degradation is enzymatic and how much is accounted for by the presence of nonenzymatic protein and of electrolyte.

In the case of desoxyribosenucleic acid degradation by tissue extracts, the effect is more easily interpreted because of the inability of salts alone to effect a desaggregation of the particles to form dialyzable components. As illustrated in Fig. 16, the degradation of the nucleate to dialyzable components by the tissue extract is slight in the absence of added electrolyte. The acceleration provided by the added salts is proportional to the concentration and is related to the valence of the cation. As in the case of purified desoxyribonuclease, maxima are observed in salt activation of tissue extracts beyond which salts have an actual depressing effect. These have not been observed in experiments with ribosenucleic acid. Figure 17 illustrates this phenomenon in an extract of rat kidney under conditions identical for those described in the previous experiments on the salt activation of tissue degradation of desoxyribosenucleic acid.

Effect of Bicarbonate and Fluoride

Greenstein, Carter, Chalkley, and Leuthardt (1946) found that the ability to desaminate and dephosphorylate desoxyribosenucleic acid in tissue extracts, which was destroyed by dialysis and restored by electrolytes, was inhibited by fluoride and bicarbonate ions, and the salts of these ions failed to restore activity in contrast with other electrolytes (with the exception of beryllium). The influence which these ions have upon the formation of dialyzable components in nucleate digests is shown in Table 12. In this experiment rat liver was extracted with 15 volumes of water; 2 cc. of the extract was added to a solution inside the cellophane bag containing 1 mg. of nucleate (1 cc. of a 0.1% solution). All salts were present in a concentration of 0.03 N. It is noteworthy that neither bicarbonate nor fluoride has an appreciable inhibitory effect on ribosenu-

TABLE 12.—INFLUENCE OF BICARBONATE AND FLUORIDE ON FORMATION OF DIALYZABLE COMPONENTS

Tissue extract	Dialyzable components											
	None		NaCl		NaCl+$NaHCO_3$		NaCl+NaF		NaF		$NaHCO_3$	
	RNA[1]	DRNA[2]	RNA	DRNA	RNA	DRNA	RNA	DRNA	RNA	DRNA	RNA	DRNA
	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
Liver	50.0	37.5	50.0	50.0	49.0	3.0	85.0	47.5	70.0	49.0	71.0	17.5
Dialyzed Liver	65.0	12.5	60.0	45.0	55.0	2.5	80.0	55.0	85.0	50.0	59.0	3.0

[1] Ribosenucleic acid.
[2] Desoxyribosenucleic acid.

cleic acid degradation. This may be a reflection of non-enzymatic desaggregation of the ribosenucleic acid. However, bicarbonate ion suppresses the degradation of desoxyribosenucleic acid in fresh-tissue extracts and fails to restore enzymatic activity to dialyzed extracts. This effect is largely independent of pH. Extracts, dialyzed or undialyzed, in the presence of bicarbonate ions at this concentration also fail to produce ammonia and inorganic phosphate when incubated with desoxyribosenucleic acid, whereas under these conditions ribosenucleic acid digests yield ammonia and inorganic phosphate

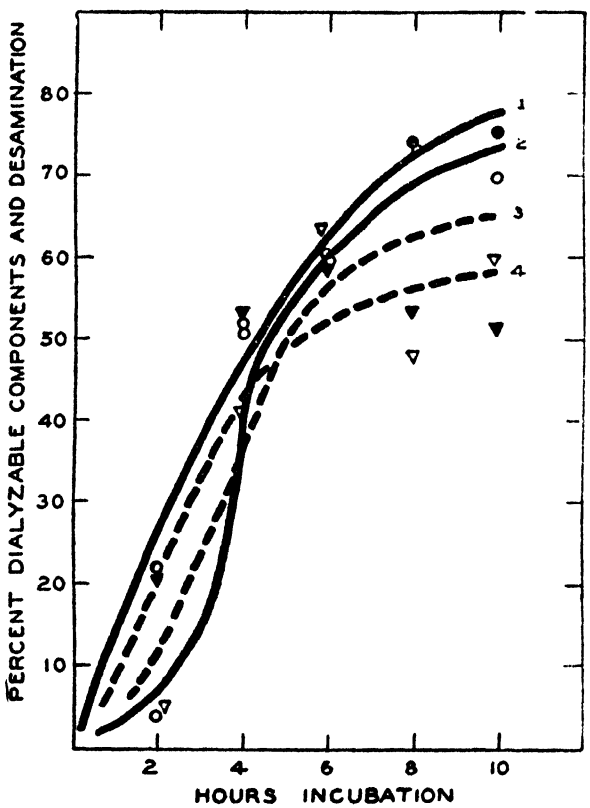

FIG. 18. Relation of the formation of dialyzable components to the liberation of ammonia in nucleate (5-mg.) digests with spleen extract (6 volumes water) incubated within cellophane bags while dialyzing against 0.01 N. sodium chloride. Curve 1, formation of dialyzable components from ribosenucleic acid; curve 2, formation of dialyzable components from desoxyribosenucleic acid; curve 3, formation of ammonia from desoxyribosenucleic acid; and curve 4, formation of ammonia from ribosenucleic acid.

(Carter and Greenstein, 1946a). Fluoride ions do not inhibit the formation of dialyzable components from either ribosenucleic or desoxyribosenucleic acid digests; in the concentration employed (0.03 N) desamination and dephosphorylation in both ribosenucleic and desoxyribosenucleic acids are practically completely inhibited.

RELATION OF DESAMINATION AND DEPHOSPHORYLATION TO THE FORMATION OF DIALYZABLE COMPONENTS

In Figs. 18 and 19 the formation of ammonia and inorganic phosphate from digests of nucleic acids with tissue extracts is compared with the rate of formation of dialyzable components. It is apparent that desamination and formation of dialyzable components proceed at almost equal rates through-

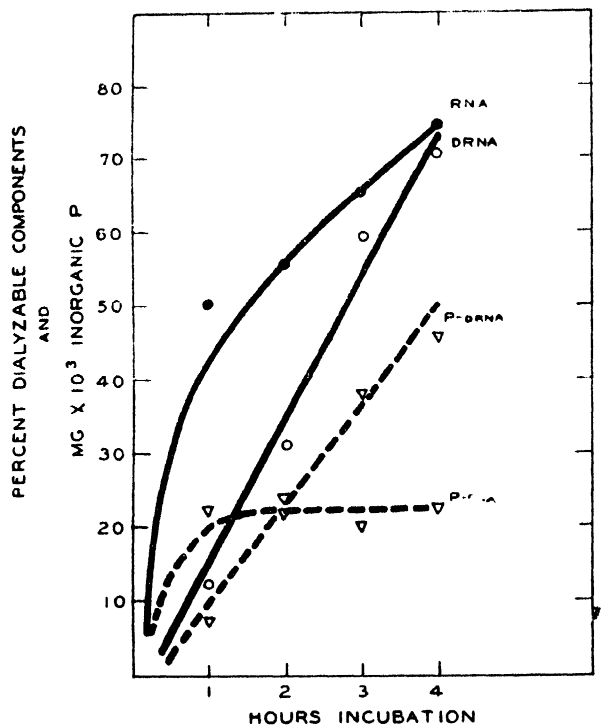

FIG. 19. Relation of the formation of dialyzable components to the liberation of inorganic phosphate in nucleate (1-mg.) digests with spleen extract (30 volumes water) while dialyzing against 0.024 M magnesium chloride. Solid lines represent dialyzable components; broken lines, inorganic phosphate.

out the course of the reaction and that no priority to either process can be assigned on the basis of such kinetic data. The relation of dephosphorylation to formation of dialyzable components under the conditions illustrated in Fig. 19 is of a different nature, in that an early maximum in the appearance of inorganic phosphate in the dialyzates of ribosenucleic acid digests is encountered, and this represents about 40% dephosphorylation of the dialyzable components. Desoxyribosenucleic acid is more completely dephosphorylated under these conditions, reaching values comparable with about 80% dephosphorylation of the dialyzable components.

From these data it would appear that one of the reactions might be rate-limiting and that upon its completion the others proceed rapidly. The data of

Schmidt, Pickels, and Levene (1939) which showed that highly polymerized desoxyribosenucleic acid was not a substrate for purified nucleophosphatase suggested that the preliminary depolymerization might be the rate-limiting reaction. Because of the comparatively small particle size and low degree of polymerization of ribosenucleic acid, it is impossible to subject the ribonuclease system to such an analysis. However, the degradation of desoxyribosenucleic acid is susceptible to experimental analysis by virtue of the following: (1) The fact that the dialyzed tissue extract is not capable of degrading desoxyribosenucleic acid to dialyzable components or forming ammonia and inorganic phosphate from the nucleate; and (2) the purified desoxyribonuclease, which is capable of degrading desoxyribosenucleic acid in the presence of proteinate, cannot desaminate or dephosphorylate the nucleate or its components. The application of these data to dialyzed spleen with or without purified enzyme was tested. Rat spleen was extracted with 30 volumes of water; 2 cc. of the extract was added to 1 mg. of desoxyribosenucleic acid in 1 cc. within the cellophane bag of the dialysis set-up. The incubation period was 4 hours. The data are given in Table 13.

TABLE 13. FORMATION OF DIALYZABLE COMPONENTS, AMMONIA, AND INORGANIC PHOSPHATE FROM DIGEST OF DESOXYRIBOSENUCLEIC ACID WITH DIALYZED SPLEEN EXTRACT

Substance	Ammonia	Inorganic phosphate	Dialyzable compounds
	Milligrams	Milligrams	Per cent
Extract	0	0.001	5
Extract plus desoxyribonuclease[1]	.035	.054	65

[1] Solution in bag contained 0.2 mg. purified enzyme.

These data show that the dialyzed tissue extract which is incapable of forming dialyzable components, ammonia or inorganic phosphate from desoxyribosenucleic acid, can, in the presence of purified desoxyribonuclease, which degrades the nucleate into dialyzable components but has no desaminating or dephosphorylating activity, form ammonia and inorganic phosphate in amounts corresponding to almost complete desamination and dephosphorylation of the dialyzable components.

That desamination and dephosphorylation are distinct from the reactions which account for formation of dialyzable components from desoxyribosenucleic acid can also be shown from experiments in which sodium fluoride in concentrations of 0.03 N was capable of completely inhibiting dephosphorylation but had an activating effect upon the formation of dialyzable components.

Ribosenucleic and desoxyribosenucleic acid are both degraded to dialyzable components by tissue extracts and purified nucleases, and in both instances the process is influenced by electrolytes. In the case of ribosenucleic acid, degradation to dialyzable components may be induced by electrolytes alone and probably by the nonenzymatic proteinates of tissue extracts, which are electrolytes of large particle size. The effect caused by the latter is difficult to evaluate because of the heat-stable nature of ribonuclease. It is known that salts are capable of inducing striking alterations in the physical properties of desoxyribosenucleic acid. However, the changes are not sufficient to produce dialyzable components from the intact nucleate. Hammarsten (1924) described changes in osmotic pressure and viscosity of solutions of desoxyribosenucleic acid induced by electrolytes. That these changes induced by electrolytes involve alterations in size and shape of the desoxyribosenucleic acid particle is illustrated in the parallel decrease in structural viscosity and streaming birefringence (Greenstein and Jenrette, 1941).

Purified desoxyribonuclease was shown in experiments described by McCarty (1946) and in this paper to be dependent upon ion activation. We found that a variety of electrolytes including organic radicals (arginine and glutamate) and sodium egg albuminate as well as ions not encountered physiologically (Carter and Greenstein, 1946a) are capable of activating the enzyme. It is possible to view this ion effect in two ways, namely, (1) that the ions have a direct activating effect on the enzyme systems, and (2) that this influence is basically due to a common effect which they exert upon desoxyribosenucleic acid in solution by virtue of their behavior as charged particles, while valence and nature of the ion affect the rate of the reaction. Under this influence, the physical properties of the nucleate are so altered as to increase its susceptibility to enzymatic attack. At the present time we are inclined to favor the second of these alternatives.

Experiments reported herein demonstrate that ribosenucleic acid and desoxyribosenucleic acid are degraded by the specific nuclease many times more rapidly in the presence of electrolyte than in salt-free solutions. This effect is best demonstrated by employing the liberation of acid groups as criteria for enzyme activity, rather than the formation of dialyzable components, for salts alone produce a desaggregation of ribosenucleic acid with the formation of dialyzable components. The primary nature of the salt effect may be the same in the case of both nucleates, i.e, a desaggregation or opening up of the molecule under the influence of electrolytes.

The problem of determining the extent to which the degradation of ribosenucleic acid in tissue extracts is independent of specific enzymatic activity is complicated by the heat-stable nature of the enzyme involved thereby making it impossible to inactivate selectively the enzyme by heat. The ability of the tissue proteinates to behave as charged particles also may account for the degradation of the nucleate to dialyzable particles. Dialysis has been shown to

TABLE 14. DECOLORIZATION VELOCITY OF METHYLENE BLUE IN AQUEOUS EXTRACTS OF NORMAL LIVER AND OF TRANSPLANTED HEPATOMA 31 IN MALE OSBORNE-MENDEL RATS[1]

Methylene blue (millimols)	Xanthine	YNA	TNA	Decolorization rate: Liver	Decolorization rate: Heptoma
	Millimols	Mg.	Mg.	Minutes^{-1} $\times 10^4$	Minutes^{-1} $\times 10^4$
0.016...	0	0	0	—	450
	0	2.5	0	—	10,000
	0	0	2.5	—	400
0.031...	0	0	0	—	178
	0	2.5	0	—	5,000
	0	0	2.5	—	222
	1.6	0	0	—	230
	1.6	2.5	0	—	5,000
	1.6	0	2.5	—	2,500
0.062...	0	0	0	—	56
	0	2.5	0	—	3,333
	0	0	2.5	—	164
	1.6	0	0	—	56
	1.6	2.5	0	—	3,333
	1.6	0	2.5	—	1,428
0.155...	0	0	0	5,000	24
	0	2.5	0	2,500	1,666
	0	0	2.5	556	62
	1.6	0	0	3,333	33
	1.6	2.5	0	2,500	2,000
	1.6	0	2.5	1,250	909
0.31....	0	0	0	2,000	10
	0	2.5	0	1,429	833
	0	0	2.5	333	36
	1.6	0	0	1,667	20
	1.6	2.5	0	1,429	769
	1.6	0	2.5	667	58
0.62....	0	0	0	714	5
	0	2.5	0	667	370
	0	0	2.5	294	23
	1.6	0	0	769	11
	1.6	2.5	0	667	222
	1.6	0	2.5	476	24
1.24....	0	0	0	218	—
	0	2.5	0	218	—
	0	0	2.5	111	—
	1.6	0	0	250	—
	1.6	2.5	0	227	—
	1.6	0	2.5	178	—
2.48....	0	0	0	39	—
	0	2.5	0	91	—
	0	0	2.5	48	—
	1.6	0	0	121	—
	1.6	2.5	0	111	—
	1.6	0	2.5	105	—
4.96....	0	0	0	4	—
	0	2.5	0	66	—
	0	0	2.5	18	—
	1.6	0	0	14	—
	1.6	2.5	0	18	—
	1.6	0	2.5	10	—

[1] Aqueous extract of tissues equivalent to 166 mg. per cubic centimeter. Mixtures consisted of 1 cc. extract, 1 cc. dye, 1 cc. water or substrates so that total volume was 3 cc. Temperature, 25°C. Anaerobic conditions maintained throughout by employment of tubes evacuated and sealed at 18 mm. mercury. End points at 98% decolorization; pH, 7.1. Concentrations given are of stock solutions. YNA = ribosenucleate, TNA = desoxyribosenucleate.

cause a loss of ability to degrade desoxyribosenucleic acid by tissue extracts while effecting no alteration in the break-down of ribosenucleic acid. Therefore, if a major portion of the degradation of ribosenucleic acid to dialyzable components in tissue extracts is non-enzymatic, this cannot be accounted for by the presence of inorganic salts. As suggested, we believe that the liberation of acid groups is the best criteria of enzymatic degradation of ribosenucleic acid, for, although this is accelerated by salts, salts alone cannot open up acid groups.

By employing the purified desoxyribonuclease, it is possible to show that desamination and dephosphorylation of desoxyribosenucleic acid is dependent upon a preliminary degradation of the highly polymerized nucleate to dialyzable components. Schmidt, Pickels, and Levene (1939) showed that the highly polymerized preparations of desoxyribosenucleic acid were not substrates of purified "nucleophosphatase," while preparations possessing low degrees of polymerization were readily dephosphorylated. We are unable to estimate the particle size susceptible to desamination and dephosphorylation other than that it is dialyzable through cellophane. The ability of fluoride to accelerate the enzymatic degradation of nucleic acids to dialyzable components while inhibiting dephosphorylation of these compounds emphasizes the independence of these two processes.

In view of the foregoing observations, it appears that the degradation of nucleic acids (desoxyribosenucleic in particular) may involve (1) a nonenzymatic desaggregation induced by a variety of electrolytes, both inorganic and organic, which increases the susceptibility of the nucleate to subsequent, (2) enzymatic splitting of certain of the internucleotide linkages with liberation of acid groups bound in organic phosphate esters. In the case of desoxyribosenucleic acid preparations that we have investigated, this phase of the enzymatic degradation accounts for

the formation of dialyzable components (ribosenucleic acid may be desaggregated to dialyzable components by salts alone) and precedes the (3) enzymatic desamination and dephosphorylation of dialyzable nucleic acid components of otherwise undetermined size. This stage in fresh tissue extracts is inhibited by fluoride, which suggests that the specific natural activator under these conditions may be magnesium.

We wish to avoid a rigid interpretation of the complex phenomena of nucleic acid degradation but tentatively propose that the foregoing stages are involved in the process.

Effect of Nucleates on the Overall Dehydrogenase Activity of Tissue Extracts

Methylene blue when added to tissue extracts under anaerobic conditions is reduced at a rate which is proportional to the activity of the dehydrogenases in the tissues and to the proportion of available substrates. Under identical conditions, and at relatively low concentrations of the dye, aqueous rat liver extracts rapidly decolorize methylene blue, whereas the decolorization rate in rat hepatoma extracts is much slower. In the presence of ribosenucleate, the decolorization rate in liver extracts is decreased, whereas in hepatoma extracts it is considerably accelerated (Chalkley and Greenstein, 1945) (Table 14). This is also true of desoxyribosenucleate. The accelerating effect of the nucleates on the decolorization velocity in the tumor extracts is evident over a wide range of dye concentrations. The effect of ribosenucleate in increasing this decolorization rate is noteworthy.

The effect of addition of xanthine in liver extracts containing the nucleate is to shift toward higher dye concentrations that concentration of the dye at which the effect of the added nucleates changes from retarding to increasing the decolorization rate of the dye. The transition from deceleration to acceleration of decolorization requires a concentration of dye low in the case of xanthine, higher for ribosenucleate, and still higher for desoxyribosenucleate.

The effect of the nucleates on the decolorization velocity of methylene blue in hepatoma extracts or in liver extracts is that essentially due to their serving as substrates. The nucleates are degraded during the incubation to smaller particles, and are desaminated and dephosphorylated. The desamination leads to the formation of oxidizable groups on the purines which react through the various dehydrogenase systems with methylene blue. Under such circumstances, the presence of the nucleates and their eventual split products results in an acceleration in the decolorization of the dye. In fresh liver extracts, the desamination of the nucleates and their split products is much slower than in the hepatoma, and the decrease in decolorization rate in the former tissue is probably to be attributed to a competitive inhibition of these products at the surface of the dehydrogenases effective for the natural and available substrates present in liver tissue.

Addendum on the Protective Effect of Desoxyribosenucleate on the Heat Coagulation of Proteins

In connection with the enzymatic experiments described above, it was noted (Greenstein and Chalkley, 1945) that whereas aqueous extracts of various tissues immersed in boiling water produced almost immediately a coagulum of protein, such extracts previously treated with sodium desoxyribosenucleate (Hammarsten) did not yield a coagulum even after many hours of heating at 100° C and remained quite clear. It appeared that the nucleate exerted a protective action on the proteins of the extract and increased their thermal stability beyond the capacity of any agent known at present. Studies on one of the most labile of proteins, namely crystalline egg albumin, are shown in Table 15 (Carter and Greenstein, 1946b).

Table 15. Effect of Added Sodium Desoxyribosenucleate on the Heat Stability of Crystalline Egg Albumin[1]

Nucleate	Period required for coagulation
Milligrams	Minutes
None	0.2
10	>120
5	>120
2.5	>120
1.25	>120
0.63	>120
0.31	>120
0.15	>120
0.08	0.2
0.04	0.2
0.02	0.2

[1] Concentration of dialyzed protein solution was 6.6%, and was brought to the pH of the nucleate solution, 6.8, with dilute NaOH. Mixture consisted of 1 cc. of protein solution and 1 cc. of either distilled water or dialyzed nucleate solution. Temperature 98° C.

It is interesting to note that there is a sharp break in the concentration range of nucleate effective in conferring complete protection against heat coagulation. At this point, somewhere between 0.15 and 0.08 mg. of nucleate, roughly 1 mg. of the nucleate will prevent the heat coagulation of about 600 mg. of egg albumin. It is probable that below this critical concentration of nucleate, some proportion of the protein molecules is not coagulated by heat, because the turbidity of solutions containing these lower concentrations of nucleate is not quite as great as in the absence of this substance.

That there is some stoichiometric relation between the concentration of protein and of nucleate was revealed by repeating the experiments mentioned in

the tabulation with an albumin solution half as concentrated. With this diluted solution of protein, 0.08 mg. of nucleate was effective in inhibiting heat coagulation whereas 0.04 mg. of nucleate was ineffective.

Different preparations of sodium thymus nucleate were used, each with varying degrees of polymerization. The results were identical throughout. In order to prove finally that the degree of polymerization of the nucleate was unimportant in the effect noted, a solution of one preparation of the nucleate was divided into two aliquots. One aliquot was irradiated at 2537A for several hours until the viscosity greatly diminished. Both aliquots were then used as in the foregoing with identical results. Even with the highly polymerized Hammarsten preparations, the lower, still effective concentrations, are barely more viscous than water.

TABLE 16. THERMAL STABILITY AT 98° C. OF EGG ALBUMIN IN THE PRESENCE OF DESOXYRIBOSENUCLEATE AND SODIUM CHLORIDE[1]

Nucleate (in milligrams)	Sodium chloride	Period required for coagulation
	Milligrams	Minutes
None	None	0.2
10	None	>120
10	9	.2
10	6	.2
10	3	.2
10	2	>120
10	1	>120
0.15	None	>120
0.15	9	.2
0.15	6	.2
0.15	3	.2
0.15	2	>120
0.15	1	>120
None	9	[2].2
None	6	[2].2
None	3	[2].2
None	2	[2].2

[1] Concentration of protein, 6.6%. Mixtures consisted of 1 cc. protein solution plus 1 cc. of either distilled water or thymus nucleate plus 1 cc. either distilled water or sodium chloride.

[2] Heavy coagulum.

The experiments were performed in the absence of added salt. Data involving the use of added salt prior to heating are given in Table 16.

The data in Table 16 reveal that the protective action of thymus nucleate is still obtained under the present conditions when 2 mg. or less of sodium chloride is present. Above this concentration of salt, heat coagulation of the protein occurs whether 0.15 or 10 mg. of nucleate is present. The critical concentration of sodium chloride, about 3-4 x 10^{-5} mols, is thus apparently independent of the nucleate concentration. Addition of salt after heating and subsequent cooling yields no coagulation. Reheating of this mixture results in a gel.

Very few sulfhydryl groups are liberated in the protein on heating under these conditions, whether nucleate is present or not. Indeed, the amount that appears, although giving a faint nitroprusside test, is too small for accurate quantitative measurement.

In contrast with the striking effect of desoxyribosenucleate, ribosenucleate employed under similar conditions is completely ineffective in preventing the heat coagulation of egg albumin. Agar is likewise ineffective. That it is the intact form of the desoxyribosenucleate which is the effective agent, intact in the sense that while it may be highly polymerized or considerably depolymerized no primary chemical bonds have released, is shown by the fact that after digestion with the specific desoxyribonuclease of McCarty, the nucleate is no longer effective (Carter and Greenstein, 1946b). This is illustrated by the following experiment. An aqueous extract of liver was heated for 30 minutes at 98° C in the presence of desoxyribosenucleate, and then cooled. There was no evidence of coagulation. Addition of desoxyribonuclease and magnesium ions, and incubation for a short period of time, resulted in flocculation and precipitation of the proteins of the extract (Table 17). Addition of desoxyribonuclease alone, or magnesium alone failed to show this effect. Ribonuclease was also ineffective. There seems to be no doubt but that the intact desoxyribosenucleate fibres hold the denatured protein in some kind of homogeneous medium, and that when the nucleate is desaggregated by its specific nuclease, it loses this property. That the forms of depolymerization induced by ultraviolet radiation (which is still effective in preventing protein coagulation) and by desoxyribonuclease—Mg^{++} (which has lost this property) are entirely different is apparent from these results. Ultraviolet radiation opens up no new groups, whereas the nuclease action produces new acid groups (*vide infra*).

TABLE 17. SUBSTANCE ADDED TO MIXTURE:[1]

	Period for coagulation at 37° C (minutes)
Desoxyribonuclease and 0.003 M magnesium^{++}	15
Desoxyribonuclease	1,440
Magnesium^{++} (0.003 M)	1,440

[1] 1 cc. of desoxyribonuclease containing 0.1 mg. purified enzyme protein added to mixture. Magnesium sulfate added as 0.05 cc. of 0.15 M solution. The mixture consisted of 1 cc. rat-liver extract (equivalent to 166 mg. tissue), and 1 cc. of 0.5% sodium thymus nucleate. The mixture was heated for 30 minutes at 98° C, with no appearance of coagulum, and then cooled.

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Discussion

Stowell: Cytologists are pleased to obtain information from the chemist which may be applicable to observations upon cells. Dr. Greenstein's results concerning the effect of various ions upon ribonucleic acid prompts me to mention some work which was just published by Dr. Zorzoli and myself in Stain Technology *22:*51, 1947. In attempting to evaluate the histochemical ribonuclease technic for nucleic acid, tissues were fixed in 11 different types of preservatives, incubated in solutions of 4 different concentrations of crystalline ribonuclease, at various temperatures, time intervals, hydrogen ion concentrations and in 5 different types of buffer solutions.

The staining of pyronin or toluidine blue varied greatly with the type of fixation as well as with the concentration of enzyme, length of incubation and type of buffer. Although a seemingly precise loss of cytoplasmic staining, which is attributed to the removal of ribonucleic acid, was frequently observed, evidence of non-specific action was also occasionally found. After some fixations, the control buffer solutions alone removed much stainable cytoplasmic substance suggesting the importance of certain ions upon the nucleic acid as shown by Dr. Greenstein. In other instances the ribonuclease prevented the staining of nuclear as well as cytoplasmic material. The alterations in the nuclear membrane and removal of sections from the slide with higher concentrations of ribonuclease were suggestive of proteolytic action. Sometimes decreased cytoplasmic staining was associated with increased nuclear staining following treatment with ribonuclease and buffer, which can further complicate the interpretation of results.

This evidence that in some instances cytoplasmic staining can be decreased or increased by buffer solutions in the absence of ribonuclease and that the enzyme may at times prevent nuclear staining and have a proteolytic action, indicate that one must be cautious in claiming a highly specific or quantitative action of ribonuclease upon tissues until more evidence has been obtained regarding the purity and action of the crystalline enzyme not only upon solutions of nucleic acid, but especially upon the altered complex material in fixed tissues.

Some preliminary experiments with desoxyribonuclease also indicated the desirability of additional

observations and standardization of materials and technic before claiming highly specific or quantitative results with such nuclease methods.

KAUFMANN: The influence of salts in the degradation of ribose nucleic acid has also been observed in a series of experiments that we have undertaken to measure the effect of ribonuclease on various cellular components. It has been observed that the removal of ribose nucleic acid from sections of various tissues depends to a considerable extent on the type of buffer used and its concentration. Our experience in these histochemical studies parallels that described above by Dr. Stowell.

McDONALD: Salts are known to affect enzyme systems where both the substrate and the catalyst are "pure" proteins. The autocatalytic formation of trypsin from trypsinogen is accompanied by the direct transformation of part of the trypsinogen into another native protein (or proteins) which cannot be changed into trypsin by any known means and which has been termed "inert" protein. Both reactions are catalysed by trypsin and occur simultaneously. Salts can either increase or decrease the rate of each reaction independently so that the ultimate amounts of trypsin and "inert" protein formed vary with the nature and concentration of the salt used. This is due to both cation and anion; the chemical nature of the ions is more important than their valency.

COHEN: I should like to call your attention to some exceptions to the prevention of protein coagulation by heat in the presence of nucleic acid. Thymus nucleohistone containing about 40% of desoxyribose nucleic acid is readily heat denatured and the protein precipitates readily. The same is true of the plant viruses containing ribose nucleic acid.

When thymus nucleohistone is heated, the protein precipitates leaving a protein-free nucleate in the supernatant solution. The interesting thing about this nucleic acid solution is that it has a far lower viscosity than a nucleate solution of the same concentration isolated by milder methods from thymus nucleohistone. This low viscosity nucleate may be produced by heating viscous nucleate at or above 75°. The heated nucleic acid does not contain dialysable materials and it appears possible that, as in the case of the nucleic acid of tobacco mosaic virus freshly isolated after heat treatment, we have a coiled molecule. It would be most interesting to compare the enzyme susceptibilities of coiled and uncoiled substrates.

THE STRUCTURES OF NUCLEIC ACIDS

JOHN MASSON GULLAND

Important Factors in Consideration of Structure

Apart from the question of molecular size, four factors are of prime importance in considerations of the structure of a polynucleotide:

1) The natures of all the individual nucleotides;
2) The natures of the linkages between the nucleotides;
3) The relative proportions of the individual nucleotides;
4) The relative positions of the nucleotides in the polynucleotide.

It illustrates our ignorance of the structures of nucleic acids that not even one of these factors is known with complete certainty for any one polynucleotide. In this contribution these factors are discussed in detail and with illustrative reference to the ribonucleic acid of yeast and the desoxypentose nucleic acid of calf thymus, and these polynucleotides are then considered from the standpoint of macromolecular structure.

The Nature of All the Individual Nucleotides

The nucleotides which have been isolated from the hydrolysis products of nucleic acids are guanylic, adenylic, cytidylic and uridylic acids in the pentose series, and in the desoxypentose series the nucleotides derived from guanine, adenine, cytosine and thymine. A survey of the literature shows that every detail of the structures of the pentose nucleotides is now known, but the position of the phosphoryl groups in the desoxypentose nucleotides still appears to be unsettled and attention has been drawn (Gulland, Barker and Jordan, 1945) to the incomplete identification of their carbohydrate radicals.

It has often been inferred openly or tacitly that these are the sole building units of polynucleotides. That may or may not be the case, but it should not be forgotten that these are merely the compounds which have been isolated in pure state from the mixtures of products of the breakdown of large and complex molecules. The quantities which have been isolated do not approach those theoretically required on the basis of an assumed presence of equimolecular proportions of the 4 nucleotides, and no systematic search for other breakdown products seems to have been recorded. There is thus potential scope for the presence of other compounds, possibly nucleotides, and there are a few, but apparently unconfirmed, records of the isolation of such additional nucleotides.

The position may be summarised in the view that although the 8 nucleotides mentioned may be regarded temporarily as the main fission products of polynucleotides, it would be unwise at present to exclude the possibility that other units may be present. This recognition of the 8 nucleotides as forming the major part of polynucleotides does permit tentative interpretations of experimental results, such as are made in this paper.

The Natures of the Linkages between the Nucleotides

Electrometric titration of yeast ribonucleic acid and its deaminated derivative (Fletcher, Gulland and Jordan, 1944) showed that this polynucleotide contains 3 primary and one secondary phosphoryl dissociations for every 4 atoms of phosphorus, and that the amino groups of guanine, adenine and cytosine and the purine-pyrimidine hydroxyl groups of guanine and uracil are in a free, titratable state. These findings confirmed the generally accepted view that the main internucleotide linkage in this acid is a phospho-ester group.

Electrometric titration of calf thymus desoxypentose nucleic acid (Gulland, Jordan and Taylor, 1947) indicated that here also the main internucleotide linkage is a phospho-ester group. Thus, the titration curve and analytical data together show that 4 primary phosphoryl groups, 3 amino groups and 2 purine-pyrimidine hydroxyl groups for every 4 atoms of phosphorus are in a free, titratable state. It should be emphasized, however, that titration methods are not sufficiently sensitive to detect a grouping other than a phospho-ester if it occurred only once in every 10 to 20 nucleotides. It is relevant to refer here to the conclusion of Astbury (1947), based on X-ray studies, that in the molecule of sodium thymonucleate "the pattern repeats along the axis of the molecule at a distance corresponding to the thickness of 8 nucleotides or a multiple of 8 nucleotides—most probably 8 or 16 nucleotides." Thus if the point of pattern repetition were also the site of a chemical linkage other than a phospho-ester group but nevertheless involving one or more titratable groups (*e.g.*, phosphoryl, amino, or purine-pyrimidine hydroxyl), that chemical linkage might not be detectable by titration, at any rate with the present technique.

The next problem is the indentification of the sugar hydroxyl groups involved in the internucleotide linkage. In the case of the desoxypentose nucleic acid there is no ambiguity, since only the hydroxyls at positions 3 and 5 of the sugar are available, and this was confirmed in the electro-

metric titration mentioned above since no groups titrate in the range pH 12-13.5; thus there are no free sugar hydroxyls, both in every sugar radical entering into the internucleotide linkages.

In yeast ribonucleic acid, however, the hydroxyls in positions 2, 3 and 5 of the sugar are available. In order to explain the very ready fission of this acid, which occurs in cold dilute alkali, it was suggested (Levene and Tipson, 1935) that the phosphoryl in position 3 of each nucleotide is united as an ester with the hydroxyl in position 2 of the sugar of its neighbour, and postulated that phospho-ester groups in position 2 should be so much less stable than those at position 3 that in alkali they would be preferentially hydrolysed, leaving the nucleotides with the more stable phosphoryl in position 3. This hypothesis is, however, untenable since nucleotides with the phosphoryl in position 2 have been synthesised and they were not abnormally unstable towards alkali. For example, uridine-2′ phosphate (Gulland and Smith, 1947) is not hydrolysed by cold alkali and has much the same stability to hot decinormal alkali as has uridylic acid. The small difference (*e.g.*, at 8 hours, 2-phosphate 23.8% dephosphorylation, 3-phosphate 17.8%) is not in itself sufficient to explain the complete fission of an ester group at position 2 while the same group at position 3 remains unattacked. Further, phospho-ester groups in position 5 have the same order of stability towards alkali as have those in positions 2 and 3.

Thus it cannot be solely an easy hydrolysis of a phosphoryl in a particular position which is responsible for the ready fission of this polynucleotide in cold alkali. It is true that di- and tri-esters of phosphoric acid are more easily hydrolysed than are mono-esters, but that cannot afford the sole explanation because di-ester groups occur in desoxypentose nucleic acids, which are far more stable towards alkali than is yeast ribonucleic acid. Moreover, diuridine phosphate, in which the phosphoryl esterifies both hydroxyls in position 2 of the sugar, has been synthesised (Gulland and Smith, unpublished) and this substance is stable towards cold alkali. At present, therefore, which of the sugar hydroxyls are involved in the internucleotide linkage of yeast ribonucleic acid remains an open question.

The Relative Proportions of the Individual Nucleotides

It has often been assumed that all polynucleotides contain the four appropriate "normal" nucleotides in equimolecular proportions, and some experimental evidence does seem at first sight to support this view although it does not bear the test of closer scrutiny. This idea has, however, given rise to the conception of the tetranucleotide, and when this term is used to denote the presence of the nucleotides in equimolecular proportions as determined analytically, it has been proposed (Gulland, Barker and Jordan, 1945) to qualify it by the adjective "statistical."

As long as the nucleic acid molecule was believed to be small in size and to consist of only four nucleotide molecules, small divergences from the statistical tetranucleotide ratio could be ignored as arising probably from experimental error or traces of impurity in the material being examined. It is obvious, however, that in a large polynucleotide molecule of high molecular weight deviations, even if slight, can be of considerable significance.

TABLE 1. ANALYSES OF DESOXYPENTOSE NUCLEIC ACID OF CALF THYMUS

	Found in material dried at 110° C and 0.1 mm. pressure	Theory*	Theory**
C	35.5% 35.5% 35.3%	35.4%	35.4%
H	3.68% 3.83% 3.56%	3.4%	3.4%
N	15.3% 15.3% 15.4%	15.9%	15.5%
P	9.35% 9.32% 9.32%	9.4%	9.4%
Na	6.9% 6.8% 7.1%	6.95%	6.98%
Ratio Purine N/ Pyrimidine N	1.60	2.0	1.6

* Theory for a large polynucleotide consisting of tetrasodium salts of statistical tetranucleotides.

** Theory for a large polynucleotide consisting of tetrasodium salts of tetranucleotides containing 1 molecule each of guanine and thymine, 1.2 molecules of cytosine, and 0.8 molecule of adenine.

Yeast ribonucleic acid has often been regarded as a statistical tetranucleotide, since the results of various methods of analysis show that purine and pyrimidine nucleotides are present in approximately equal amounts. These methods depend in general on the facts the purine nucleotides are rapidly dephosphorylated and have their sugar easily converted into furfural by acid, whereas such changes take place only slowly and with difficulty in the pyrimidine nucleotides. Such procedures are only moderately accurate and therefore give only approximate answers. The precipitation of purines, but

not pyrimidines, by means of silver sulphate (Schmidt and Levene, 1938) after hydrolysis in sulphuric acid solution has been shown to be quantitative, and when this fractionation was applied to the acid hydrolysates of yeast ribonucleic acid and nitrogen was estimated in the two fractions (Gulland, Jordan and Threlfall, 1947), the average ratio Purine N/Pyrimidine N was 1.86 instead of the theoretical 2 for a statistical tetranucleotide. This may mean either that there are more pyrimidine than purine nucleotides, or that there are the same numbers of the two types but that some of the uridylic acid is replaced by cytidylic acid, which contains 50% more nitrogen. The latter alternative seems the less likely, since the titration data for yeast ribonucleic acid and its deaminated derivative indicate (through titration of purine-pyrimidine hydroxyls) the presence of one guanine and one uracil radical for every 4 atoms of phosphorus.

When the silver sulphate method was applied to hydrolysates of the tetra-sodium salt of desoxypentose nucleic acid of calf thymus, the average ratio Purine N/Pyrimidine N was 1.60 instead of the theoretical 2 for the statistical tetranucleotide. This figure may be linked with the electrometric titration data for the same desoxypentose nucleic acid and for the barium thymate prepared from it by mild acid hydrolysis (Gulland, Jordan and Taylor, 1947). Analysis of these titration curves reveals that for every 4 atoms of phosphorus there is one radical of guanine, one of thymine, about 1.2 of cytosine and about 0.8 of adenine. Table 1 shows that the electrometric titrations and analytical results are in agreement, and consideration of both sets of data suggests that the desoxypentose nucleic acid does not conform to the statistical tetranucleotide ratio.

The Relative Positions of the Nucleotides in the Polynucleotide

As long as nucleic acids were thought to be composed of four nucleotide molecules only, the definition of the relative positions of those four molecules was apparently a relatively simple problem, and several determinations are recorded showing which nucleotides are liberated first on partial hydrolysis on the assumption that these would be terminal in the chain.

With the recognition of polynucleotide character the problem opened at once on a new, and difficult, phase, but some investigators concluded immediately that the large polynucleotides are polytetranucleotides formed by repeated condensation of tetranucleotides in which the individual nucleotides occur in an invariable sequence.

The evidence outlined above does not favour the poly-tetranucleotide hypothesis, and whatever may eventuate in future, there does not seem at present to be any indisputable chemical evidence that individual nucleotides are arranged in the polynucleotide in a fixed or regular sequence. At first sight this view might seem to be at variance with the evidence derived from X-ray investigation. Astbury (1947) states that it seems improbable, to judge by the degree of perfection of the X-ray film diagram, that the 4 different kinds of nucleotides are distributed simply at random, but rather must they follow one another in some definite order, at least in the more crystalline regions of the structure which give rise to the regular diffraction pattern. It is not clear from that statement, however, whether the perfection of the diagram would be upset by a shift or interchange in the positions of nucleotides (*e.g.* the 2 pyrimidine nucleotides) in parts of the chain relative to the positions in other parts, and this possibility does not seem to be excluded by Astbury's conclusion that "for the moment we must content ourselves with the statement that present X-ray evidence indicates that probably the bulk of a sodium thymonucleate fibre is constructed from molecules built to a regular pattern, geometrical or chemical or both, based on a sequence of nucleotides that is a multiple of four."

Two points are the result of these views. First, claims of the type not infrequently made that two nucleic acids are identical cannot be entertained unless account is taken of the possibilities of isomerism. Thus, two polynucleotides containing the same nucleotides in the same proportions would be distinct individuals if the positions of even two nucleotides in the chain of one of them were interchanged; they would have the same analytical composition and no doubt exhibit the same physical properties, but would nevertheless be chemically and perhaps biologically distinct. Second, nucleic acids may have extensive potentialities for specificity (Gulland, 1947; Muller, 1947) not dissimilar to those of the proteins, and if nucleoproteins are to be regarded as specifically characteristic, whether of biological groups or individuals or of viruses or tumour-producing agents, then it seems possible that both nucleic acid and protein may contribute to the specificity, and not the protein alone as has often been thought.

The Macromolecular Structures of Yeast Ribonucleic Acid and Calf Thymus Desoxypentose Nucleic Acid

After the foregoing analysis of the factors which must be investigated before the more precise details of nucleic acid constitution are known, it is appropriate to consider yeast ribonucleic acid and calf thymus desoxypentose nucleic acid from the standpoint of present knowledge of their macromolecular structures.

Yeast Ribonucleic Acid

Up to date there is no record of the examination of a sample of this acid which can be regarded as being in an undegraded state. Electrometric titra-

tion of purified commercial samples of the acid and its deaminated derivative (Fletcher, Gulland and Jordan, 1944) revealed that in the aggregate for every four atoms of phosphorus there are three primary and one secondary phosporyl dissociations, and Figs. 1, 2 and 3 were proposed as possible structures for sections of the polynucleotide chain; polycondensation would occur either through group A or B, the other member of the pair remaining singly linked, and as already stated (Gulland, Barker and Jordan, 1945) a polynucleotide of this nature provides possible explanations of the low content of phosphorus frequently recorded for yeast ribonucleic acid, and of the action of ribonucleinase which hydrolyses only about one-third of the polynucleotide (Kunitz, 1940; Zittle, 1946a) and yet releases some of each of the 4 nucleotides (Loring and Carpenter, 1943). An interesting parallel with the titration of yeast ribonucleic acid is the investigation of the pentose nucleic acid of blowfly larvae (Khouvine and Grégoire, 1944); here also electrometric titration reveals the presence of one secondary phosphoryl dissociation for every 4 atoms of phosphorous.

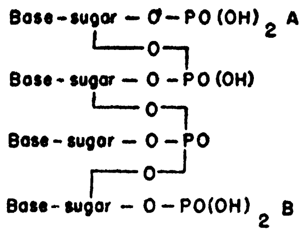

FIG. 1.

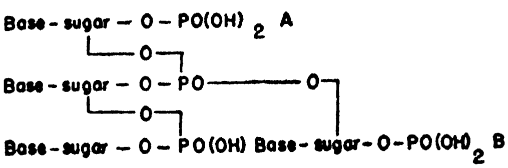

FIG. 2.

Recently Zittle (1946b) criticised the proposal of structures based on Figs. 1, 2 or 3 and favoured the straight chain formula put forward by Lèvene (Levene and Simms, 1926) although admitting that that formula does not account for the action of ribonucleinase. It would not be appropriate to enter here into a detailed discussion of Zittle's observations, but it should be noted that analysis of his titration curve for the ribonucleic acid shows the presence of about 0.7 secondary phosphoryl dissociations for every 4 atoms of phosphorus; it is an interesting coincidence that the titration curve of yeast ribonucleic acid (Fletcher, Gulland and Jordan, 1944) also revealed 0.7 secondary phosphoryl dissociation before correction for deficiency of phosphorus. It is clear that a polynucleotide of the Levene straight chain type could not contain such a high content of secondary phosphoryl dissociation unless the average chain length were only 6 nucleotides, and there is no suggestion of that in the paper. Consequently, for this and other reasons it is maintained that Zittle's criticism of structures of the type in Figs. 1, 2 or 3 is not justified.

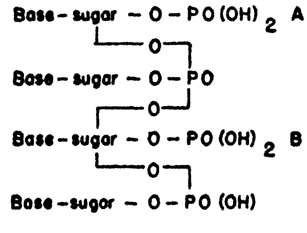

FIG. 3.

Calf Thymus Desoxypentose Nucleic Acid

A sample of the tetrasodium salt of this acid was prepared (Gulland, Jordan and Threlfall, 1947) using essentially the extraction procedure of Mirsky and Pollister (1942, 1943) and the deproteinisation technique of Sevag, Lackman and Smolens (1938); during the preparation the reaction did not vary significantly from pH 7. This extremely fibrous material was shown (Creeth, Gulland and Jordan, unpublished) to be electrophoretically homogeneous, and when examined by Cecil and Ogston (unpublished) in the ultracentrifuge was again found to be homogeneous and to have a molecular weight of about three million and an axial ratio of 710.

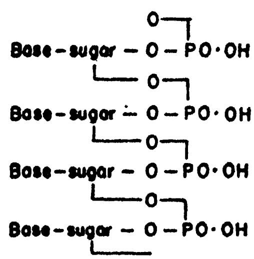

FIG. 4.

When the tetrasodium salt in aqueous solution at pH 7 was titrated electrometrically with acid or alkali (Gulland, Jordan and Taylor, 1947), no groups were titrated at first between pH 5 and pH 11 but outside those limits there occurred a rapid liberation of groups titrating in the ranges pH 2-6 and pH 9-12 respectively. On back-titration either with acid from pH 12 or with alkali from pH 2.5, a

curve was obtained which was different from that representing the initial titration, and it is significant that the same curve was obtained whether the back titration was with alkali from pH 2.5 or with acid from pH 12. Subsequent titration of the solution followed the second or back-titration curve, which is thus the true titration curve of the polynucleotide. It was concluded that the groups titrated between pH 8 and pH 12 were the hydroxyl groups of guanine and thymine, and that those titrating in the range pH 2.5-6.3 were the amino groups of guanine, adenine and cytosine. The amount of secondary phosphoryl dissociation, if present, was extremely small, but the ratio of sodium to phosphorous showed that 4 phosphoric acid dissociations were present; these must therefore have been primary. Thus the back titration curve coincided almost exactly with the theoretical curve for an unbranched polynucleotide, Fig. 4; the type of structure originally proposed by Levene (Levene and Bass, 1931) represents a section of the polynucleotide chain.

The complete idenity of the back titration curves suggested that acid and alkali have the same effect in liberating amino and hydroxyl groups, and the simplest explanation of the effect described is that in the original sodium salt of the desoxypentose nucleic acid a linkage occurs between hydroxyl and amino groups in the form of hydrogen bonding, involving resonance between the two hybrid forms $-NH_3^+$ O′- and $-NH_2$ HO- and possibly including water molecules as part of the bonds. Hydrogen bonding between acidic and basic groups tends to break down when these groups are neutralised, which is in conformity with the observed liberation of groups on treatment of the polynucleotide with alkali and acid.

For steric reasons this hydrogen bonding cannot exist between the amino and hydroxyl groups of the same nitrogenous radical, but must occur between nucleotides of the same or adjacent covalent chains. Light was shed on this point by studies of the viscosities of solutions of the tetrasodium salt (Creeth, Gulland and Jordan, 1947). Aqueous solutions were strongly birefringent, and extremely viscous; comparative values for relative viscosities of thymus desoxypentose nucleic acid at pH 7 and 25° are 5.7 for a 0.3% solution measured in an Ostwald viscometer (Vilbrandt and Tennent, 1943), 5.53 as a limiting value at high pressures for a 0.25% solution in a capillary viscometer (Greenstein and Jenrette, 1940), and 116 for a 0.243% solution in a capillary viscometer at a pressure of 8000 dynes/cm^2 (Creeth, Gulland and Jordan, 1947).

When the solution of the polynucleotide is made acid or alkaline the viscosity and streaming birefringence decrease (Hammarsten, 1924; Vilbrandt and Tennent, 1943), and Creeth, Gulland and Jordan (1947) have shown that in the variation of viscosity with change of the pH of the solution there are two highly critical pH values for their samples of this polynucleotide. The initial high viscosity of the solution at pH 7 is fully maintained as the pH is varied between 5.6 and 10.9, but in solutions more acid than pH 5.6 or more alkaline than pH 10.9, the viscosity falls sharply to a very low value at which it no longer varied with the applied pressure. It is significant that these critical values of pH coincide with those observed for the liberation of acid and basic groups in the titration.

It was suggested (Vilbrandt and Tennent, 1943) that the decrease in viscosity brought about by the addition of acid or alkali is caused by a depolymerisation of the desoxypentose nucleic acid, and the nature of this change now becomes clearer. The change cannot involve any extensive rupture of covalent internucleotide linkages since no increase in the amount of secondary phosphoric acid dissociation was observed in the back titration curve (Gulland, Jordan and Taylor, 1947). Nevertheless the fall in viscosity and streaming birefringence is obviously closely associated with the fission of hydrogen bonds and the liberation of the amino and hydroxyl groups which are mutually blocked in the original sodium salt of the nucleic acid. It is thus evident that at least a large proportion of the hydrogen bonds unite nucleotides in neighbouring polynucleotide chains; the fission of hydrogen bonds between nucleotides in the same chain could not account for the loss of streaming birefringence and structural viscosity if the molecule retained a straight linear form. The large number of cross-linking hydrogen bonds which are possible, the maximum being 2 for every 4 atoms of phosphorous, would give a considerable degree of stability to the polynucleotide micelle, and in order to degrade it the simultaneous breaking of many of the hydrogen bonds might be necessary. Such a process would lead to an abnormal titration curve of the type given by the tetrasodium nucleate as first isolated. It is thus in conformity with the titration, viscosity and streaming birefringence data to regard the micelle of the original tetrasodium nucleate as disaggregating into smaller units by rupture of the hydrogen bonds joining them, and it would seem probable that the molecular species in solution at pH 2.5 or pH 12.5 represent what is generally referred to as the desoxypentose polynucleotide comprising nucleotides covalently linked; this hypothesis may be compared with that put forward by Cohen and Stanley (1942) to explain the behaviour of the ribonucleic acid of tobacco mosaic virus.

The viscosity of a solution of the sodium salt of thymus desoxypentose nucleic acid is reduced by the addition of neutral salts (Greenstein and Jenrette, 1940, 1941). With the high molecular weight polynucleotide, Creeth, Gulland and Jordan (1947) found that this reduction was considerable even at low salt concentrations, and observed a critical concentration (approximately 0.01 M in the case of

sodium chloride), such that any increase of the concentration above the critical produced relatively only a small change in the viscosity; a slight maximum was noticed at approximately 1 M sodium chloride, a result similar to that observed by Needham, Kleinzeller, Miall, Dainty, Needham and Lawrence (1942) for the action of salts on the viscosity of myosin solutions. The observations of Greenstein and Jenrette were made at salt concentrations greater than the critical, and they suggested a reversible depolymerisation as the explanation of the fall in viscosity on the addition of neutral salts, the guanidine ion being particularly effective. By titrating the tetrasodium nucleate in the presence of 1 M potassium chloride or guanidine sulphate (1 M with respect to the guanidine ion) Gulland, Jordan and Taylor (1947) showed, however, that even in the presence of these salts blocking of the purine-pyrimidine hydroxyl groups persisted, the initial alkaline and back titration curves following the corresponding curves for titrations in absence of added neutral salts. The change in the viscosity on the addition of neutral salt thus bears no relationship to the change which occurs on the addition of acid or alkali. It could be attributed to any of at least three processes, *viz.* a disaggregation of aggregates of micelles, changes in the shape of the micelle, or changes in the ion atmosphere and hydrosphere, but the data so far obtained do not permit a choice between alternatives.

It is evident that information is accumulating on the properties and structure of thymus desoxypentose nucleic acid and of polynucleotides from other sources, but it is by no means clear to what extent such observations on polynucleotides which have been isolated are applicable to polynucleotides in the native state. Cohen (1945), for example, observed that the viscosity of a solution of thymus nucleohistone increases as the protein is removed by the enzymes chymotrypsin and trypsin and suggested that the nucleic acid molecules aggregated when set free from the nucleoprotein. If aggregation does in fact occur in those conditions, it raises the problem as to whether the mode of separation of the nucleic acid from the protein, *e.g.* by precipitation of the nucleic acid, by concentration of the protein at an interface, by an enzyme degradation of the protein or by electrophoresis, may have an influence on the particular form of aggregation (Gulland and Jordan, 1947). Polynucleotide chains undoubtedly do aggregate, because when a solution of the sodium salt of thymus desoxypentose nucleic acid is allowed to stand at pH 7 after treatment with alkali at pH 12 there is a gradual increase in the viscosity (Vilbrandt and Tennent, 1943; Creeth, Gulland and Jordan, 1947) which after 96 hours approaches that of the original solution of nucleic acid at pH 7. It would appear, however, that the two micelles may not be identical; the viscosity of the new solution at pH 7 has a different structural character than that of the original, being less at high pressures and greater at low pressures, and the titration of the new micelle does not follow the same course as the titration of the original tetrasodium nucleate before treatment with alkali. Such results emphasise the complexity of the problems, but are also the fingerposts for future research.

The author welcomes this opportunity of expressing his warm gratitude to the Long Island Biological Association for its invitation to participate in this Symposium and for its generosity in making that possible.

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DISCUSSION

COHEN: Asymmetry estimates for desoxyribose nucleic acid have been mentioned several times in the course of the Symposium, and I should like to point out that an accurate estimate for this material has probably not yet been made. Estimates of axial ratio of a molecule may be calculated from viscosity data, sedimentation and diffusion data, etc., using certain assumptions. It has been recognized by all workers that these data may be so applied only if they are corrected for particle interaction, involving extrapolations to infinite dilution. In the case of sedimentation and diffusion data for this nucleic acid the values vary markedly with decreasing concentration, with the result that, so far as I know, accurate extrapolations to infinite dilution have not yet been made.

The estimation of axial ratio from viscosity data by the Simha equation, for example, employs a function termed intrinsic viscosity. This requires a linear relation between specific viscosity and concentration in the lowest concentration ranges studied. Since "native" thymus nucleic acid shows a viscosity-concentration curve of increasing slope, it has not been possible to get reliable estimates of intrinsic viscosity on that material.

Briefly, all reported data on thymus nucleic acid in viscous solutions describe molecular aggregates and accordingly asymmetry estimates of the nucleic acid molecule have been without foundation. It was possible to make estimates of the particle shape of the nucleic acid of tobacco mosaic virus only because, for some unknown reason, particle interaction was practically negligible. Thus sedimentation and diffusion data varied only slightly with concentration, while the viscosity-concentration relation was linear throughout the entire range studied.

I should like to ask Dr. Gulland one question. In your experiments with ribose nucleic acid and viper's venom, the appearance of alkali-labile nucleotides was suggested to signify the formation of ribose-2-phosphate nucleotides. In view of the alkali-stability of ribose-2-phosphate nucleotides which you describe, is any other interpretation to your previous data suggested?

GULLAND: In the paper referred to by Dr. Cohen (Gulland, J. M., and Walsh, E. O'F., J. Chem. Soc. 172-175, 1945), a process of exclusion of different groups in the yeast ribonucleic acid molecule seemed to leave the hydroxyls at C_2 of the sugars as the only possible sites of the alkali-labile phosphoryl groups which were revealed by the action of Russell's viper venom. This view was based on the theory propounded by Levene and Tipson that phosphoryl groups at C_2 would be inherently labile towards alkali; but we pointed out that it could not be accepted unreservedly until positive, rather than negative, evidence becomes available, since so far as we were aware the literature does not record, for substituents in the hydroxyls of the sugars, differences in stability so marked in degree as to explain the complete fission of an ester at C_2 whilst a similar linkage at C_3 remains wholly unattacked. No clear interpretation of the results can be suggested at present on chemical grounds, although one can fall back on speculations such as have been considered by Gulland and Smith (1947) or by Astbury (1947). It should be noted that there is no theoretical reason why all the phosphoryl diester groups in yeast ribonucleic acid should occur uniformly at the same carbon atoms (*e.g.*, all 2-3 or all 3-5) in all the sugar molecules, and it would seem that a classical method of attack, which is now in progress, may throw light on this question—namely, methylation of the nucleic acid, hydrolysis and identification, and if possible estimation, of the partially methylated sugars. The difficulties of such procedures are, however, recognized.

ZITTLE: I would like to comment on Dr. Gulland's reference to my titration of ribonucleic acid. The titration in question was prepared for comparison with a titration of the nucleic acid after it had been completely hydrolyzed, judged by the liberation of phosphoric acid, with a phosphoesterase preparation from calf intestinal mucosa. Comparison of the two titration curves (a graph of the

curves was shown) shows that 3.4 secondary phosphoric acid groups are released by hydrolysis for every four phosphorus atoms of nucleic acid. It was from these data that Dr. Gulland's structure for ribonucleic acid, which would require the release of 3 secondary and 1 primary phosphoric acid groups, was questioned. The acid groups released on complete hydrolysis were also measured from the amount of sodium hydroxide required to restore the hydrolysate to its initial pH and also manometrically from the gas released when the reaction was performed in a bicarbonate medium. These procedures would measure total acid groups released. Since the acid measured was in agreement with the secondary phosphoric acid groups determined by titration it was concluded that no primary phosphoric acid group was released which is also in disagreement with the proposed structure for ribonucleic acid. It is clear from what Dr. Gulland has said that a simple straight chain structure for ribonucleic acid is untenable also.

GULLAND: As stated in my communication, I am unwilling to enter here into a detailed discussion of Dr. Zittle's observations as recorded in his paper, but I am glad to note that he agrees that a simple straight chain structure for yeast ribonucleic acid is not admissible if its titration curve reveals the presence of a high proportion of secondary phosphoryl dissociation, approaching one group per four phosphorus atoms in the aggregate. I hope to examine the details of Dr. Zittle's paper more fully than has yet been possible. At the same time, it is now evident that in any considerations of the titration of samples of yeast ribonucleic acid the findings of Chantrenne (Chantrenne, H., Bull. Soc. Chem. Biol. *28:* 465, 1946) must be carefully examined. This author fractionated yeast ribonucleic acid into two fractions of different solubility, one having pK values of 2.3, 3.7, 4.2, 6.0, 10.1, 10.2, and the other of 3.7, 4.2, 6.2, 8.6, 10.1, 10.2.

WEISSMAN: Have you ever measured the optical rotation of different nucleic acid samples to see whether variations within the "macromolecule" actually occur? Also, could you not use the optical rotatory power as a means of checking shifts in the sugar linkages, in a fashion similar to that used in measuring mutarotation of sugars?

GULLAND: We have not as yet examined systematically different samples of nucleic acids by polarimetric methods with the aims outlined by Dr. Weissman, having been hesitant to embark on such studies, partly in view of the difficulties in interpreting the nature of variations in optical rotation, if such should be observed, and partly on account of pressure of work on other aspects of nucleic acid chemistry.

POLLISTER: Dr. Gulland's studies of the properties of the thread-like molecules of desoxypentose nucleic acid are of considerable cytological interest. The chromosome consists of a chromonema thread which at times becomes coiled in a quite regular helical fashion. Study of larger chromosomes has shown that the gyres of this major helix are in turn coiled in a minor helix, and, in cases most favorable for observation, a third order of helix has been found. This last is at the arbitrary limit imposed by the resolving power of the microscope. It has often been suggested that the hierarchy of coils actually extends downward to a "molecular spiral."

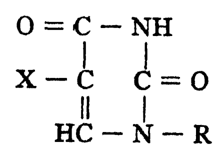
Since desoxypentose nucleic acid is the only known chemical component of the chromosome that has been demonstrated to be of linear form and to be capable of forming a fibre, it is our best hope to look to the properties of desoxypentose nucleic acid in a search for the chemical basis of the molecular spiral which is supposed to be reflected in the visible spiral.

Would Dr. Gulland care to comment on the possibility that the thread-like molecule of desoxypentose nucleic acid can be thrown into a regular helix by the development of evenly spaced linkages, perhaps hydrogen bonds, between different points along the molecule? May not some of the data showing a change of axial ratio perhaps be interpreted as the result of regular helical coiling to form a shorter and thicker thread, without any actual change in molecular weight?

GULLAND: It does seem possible that the data showing a change of axial ratio on treating the sodium salt of calf thymus desoxypentose nucleic acid with alkali or acid could be interpreted by a marked change in molecular shape without change in molecular weight, rather than by splitting of a long micellar structure into shorter chains of covalently linked nucleotides, or alternatively both types of change might occur simultaneously. It may eventuate that the electrophoretic and ultracentrifuge studies now in progress will shed light on the interesting and stimulating suggestion of Dr. Pollister.

GOODMAN: At the University of Colorado we have been working on a synthetic approach to the structure and functions of the nucleic acids. Drs. Donald Visser, Karl Dittmer and I have embarked on a long path involving tedious syntheses and preparations.

We have synthesized a number of nucleosides in which the following sugars have been substituted in the 1-position of the pyrimidine ring: D-glucose, D-galactose, D-arabinose and D-ribose. The following structure illustrates the compounds produced:



X = H, $-CH_3$, Cl, Br

R = D-glucose, D-galactose, D-ribose, D-arabinose

These compounds show the following physical properties:

Nucleosides	M.P.	$[\alpha]_D^{25}$ C = 2.0	Ultraviolet Absorption A Maximum	Ultraviolet Absorption A Minimum
1-D-Arabinosyluracil	252	−88.4	2580	2280
1-D-Galactosyluracil (1)	251	+59.7	—	—
1-D-Glucosyluracil (2)	209	+21.0	2580	2280
1-D-Ribosyluracil (3)	257	−139.2	2620	2300
1-D-Arabinosylthymine	280	−0.1	2640	2320
1-D-Galactosylthymine	130-40	+49.7	—	—
1-D-Glucosylthymine	269	+14.6	2640	2340
1-D-Ribosylthymine	252	−110.0	2660	2340
Natural Uridine	165	−4.0	2630	2310
Arabinosyl-5-chlorouracil	266	−50.4	2740	2380
Glucosyl-5-chlorouracil	280	+13.9	2770	2360
Ribosyl-5-chlorouracil	245	−87.3	2770	2360
Arabinosyl-5-bromouracil	264	−27.7	2760	2410
Glucosyl-5-bromouracil (2)	263	+10.9	2760	2410
Ribosyl-5-bromouracil	230(D)	−61.8	2790	2420

These nucleosides have been and are being tested for possible metabolite antagonist action on various micro-organisms, including *E. coli* (A.T.C.C. 9723), *L. casei* (A.T.C.C. 7469) and Neurospora (1298 A and 1298). If carried to their logical conclusion, these syntheses should go far to help in the elucidation of the structure of nucleic acids and should also be valuable tools in arriving at an understanding of the metabolic role of the nucleic acids and their derivatives.

It should be profitable to study these unnatural nucleosides as possible virus inhibitors, anti-carcinogens, or even mutagenic substances in bacteria and tissues.

THE NUCLEOPROTEINS IN VIRUS REPRODUCTION

HOLGER HYDÉN

All chemical analyses carried out on viruses have shown that the infective agent contains nucleoproteins as a main constituent.

Two facts are to be stressed regarding these results. Most of the chemical analyses have been carried out on plant viruses, owing to the relative ease with which large samples can be collected. About 200 different plant viruses have been described, and of these only 10 have been investigated by chemical and physical methods. They have all been shown to contain ribose nucleotides. Viruses which reproduce themselves in the cells of mammalian organisms have also been analyzed, and the finding of nucleoproteins as a main constituent seems to be consistent.

The viruses have been studied during reproduction, when they are available in sufficient amounts for macro-chemical investigations. During this period their influence on the host cells must be most pronounced and the metabolism of the host cells most affected by the virus. This raises the question of the mechanism of virus reproduction and how it affects the intracellular processes of the host cells.

The viruses reproduce themselves within the host cells, which are in most cases capable of producing nucleoproteins of their own. The studv of virus reproduction therefore requires a comprehensive knowledge of the organization of living cells with regard to the production and metabolism of nucleoproteins.

This problem has been studied by means of microspectrographic and other cytochemical methods in the department for cell research at the Karolinska Institutet in Stockholm since 1936. For description of the methods employed the reader is referred to the original papers (Caspersson, 1936, 1940a and c, 1941; Hydén, 1943a; Hamberger and Hydén, 1945). A survey of the methods is given by Dr. Thorell in this volume.

Before reporting the results on virus-infected cells, I will give a short survey of the results on the normal cell, on the basis of investigations carried out in the department of cell research of the Karolinska Institutet from 1936 to 1947.

The General Organization of the Metazoan Cell

The formation of protein molecules in general is a process of primary importance for all living matter. Irrespective of the organization of any structure, it requires the presence of nucleic acids. There is, however, a distinct difference between the type of nucleic acid taking part in the reproduction of the hereditary factors in the cell nucleus and the type required for the increase of other cell protein, whether for cell growth or the formation of, for example, cell secretions.

Regarding the nucleus, a series of observations on the processes during mitosis (Caspersson and Schultz, 1938, 1939; Caspersson, 1939, 1940, 1941) led to the conclusion that nucleic acids of the desoxyribose type are necessary for the reproduction of the gene protein, the primary life process in the cell. The euchromatin containing desoxyribose nucleic acids is characterized by a strict structural arrangement, whereas the products of the heterochromatin form more or less structureless masses. The nucleoli developing during telophase can be regarded as products of the heterochromatin (Caspersson and Schultz, 1938). The latter, far from being inert, has been shown to influence the formation of ribose nucleic acids in the cytoplasm, which nucleic acids are necessary for the cytoplasmic protein formation.

From results obtained by cytochemical methods on different material (Caspersson and Schultz, 1939a and b; Caspersson, 1939; Caspersson, Schultz and Aquilonius, 1940; Caspersson and Thorell, 1941; Landström-Hydén, 1942; Caspersson and Santesson, 1942; Hydén, 1943a and b; Thorell, 1944, 1946; Hamberger and Hydén, 1945) it has been shown that in the production of protein in *the cytoplasm* the following mechanism operates. All cells in which there occurs an intensive formation of cytoplasmic protein are characterized by large nucleoli and large amounts of ribose nucleic acids in the cytoplasm (Caspersson and Schultz, 1938, 1939; Caspersson and Nyström, 1941; Caspersson, 1941; Caspersson, Hydén and Aquilonius, 1941; Caspersson and Brandt, 1940; Caspersson and Thorell, 1941, Landström, Caspersson and Wohlfart, 1941; Landström-Hydén, 1942; Caspersson and Santesson, 1942, Hydén, 1943; Thorell, 1944; Thorell and Wilton, 1945; Hamberger and Hydén, 1947).

From the heterochromatic portion of the nucleus substances containing proteins rich in diamino acids are produced. These substances accumulate and form the main part of the nucleolus. From the nucleolus they diffuse towards the nuclear membrane, on the outside of which a production of ribose nucleic acids occurs (Fig. 4). At the same time the amount of cytoplasmic protein increases, and apparently its formation is in some way connected with the nucleic acid changes. This mechanism can be followed by direct measurements, especially in certain nerve cells, as will be shown in the following (Hydén, 1943).

The nucleolus and the ribose nucleic acids of the cytoplasm can be used as an indicator of the inten-

sity of the protein formation in the cell. The degree of activity can be determined by microspectroscopic measurements of the composition of the nucleolus and the cytoplasm.

This organization of the metazoan cell is shown to be valid also for bacteria and yeast cells (Caspersson and Brandt, 1942; Brandt, 1944; Caspersson, Malmgren, Thorell and Bjerkelund, 1945). In both bacteria and yeast cells during the period of growth —i.e., the formation of cellular protein—there is an intense production of ribose nucleic acids in the cell bodies. Both bacteria and yeast cells possess a chromosome-like organelle containing desoxyribose nucleic acids. In yeast cells, moreover, an organelle corresponding to the heterochromatic parts of the metazoan cells is found; it contains ribose nucleic acids.

Thus there is a gradual transition between the organization of the lower organisms and that of the higher organisms represented by the metazoan cell.

Experimental Studies on Viruses

In the present investigations, interest has been focused on four points:

1. Whether virus substances can be localized in cells from virus-infected animals,
2. Whether different types of virus show a specific organization comparable to the nuclear organization of the metazoan cells,
3. Whether the nucleic acid mechanism is valid also for viruses,
4. The manner in which the viruses act upon the host cells.

Of the more complex animal viruses, only a few have been subjected to thorough chemical investigation. Vaccinia virus, rabbit papilloma and influenza contain nucleic acids of the desoxyribose type (Smadel and Hoagland, 1942; Taylor, Sharp, Beard, D. and Beard, J. W., 1942; Taylor, Sharp, Beard, D., Beard, J. W., Dingle, and Feller, 1943; Stanley and Knight, 1944; Hollaender and Oliphant, 1944; Taylor, 1944), whereas chicken sarcoma, equine encephalomyelitis, and probably poliomyelitis are of the ribose type (Pollard, 1939; Taylor *et al.*, 1942; Gard, 1942).

The present investigations have been carried out with microspectrographical and other cytochemical methods on virus-infected tissues from man and other mammals. The material has been fixed according to the Altmann-Gersh freezing-drying method or in Carnoy's solution. In the present investigations, special precautions were taken regarding the cytological fixation of the virus-infected cells. Most of the investigations with microspectrographical methods were carried out on frozen-dried specimens in which the optical conditions permitted such analyses. It was found that ordinary cytological fixatives produce unfavorable changes in the material of the virus-infected cells. The unspecific light losses due to reflexion and dispersion were too great in the absorption spectra from cells thus treated to allow an analysis of them. The preparations have been cut in sections 5-10 μ thick and treated with chloroform in order to remove other substances which might contribute to the absorption around 2600 A (see Hydén, 1943). The cytochemical investigations have been carried out on individual cells, using the methods referred to in the introduction.

Viruses were selected which cause different cytological changes in the host cells, so that the results obtained might be of importance for understanding the mode of propagation of groups of viruses and serve as a background for attacking the more complicated neurotropic viruses.

I. *Examples of pantropic viruses containing desoxyribose nucleic acids*

1. *Molluscum contagiosum* is taken as an example of the more highly organized viruses, containing desoxyribose nucleotides during their reproduction, the main development taking place in the cytoplasm of the host cell (Caspersson and Hydén, 1945).

Fig. 1a is an ultraviolet photograph of the various cell layers in normal epidermis. All photographs were taken at the absorption maximum of the nucleic acids, 2600 A, under identical conditions, and have been copied on the same scale. The distribution of dark areas on the prints will therefore give a general impression of the distribution of the nucleic acids in the different regions of the cells. By a subsequent analysis with ultraviolet microspectrography the exact distribution of both nucleic acids and proteins can be determined.

All cells in the deeper layers of the epidermis are characterized by spherical or ellipsoidal nuclei, which contain proteins in moderate concentrations and small quantities of desoxyribose nucleic acids. In each nucleus a small nucleolus can be clearly distinguished by virtue of its high absorption power. The analysis shows that the nucleotides are mostly of the ribose type. Only at the periphery of the nucleolus can there be found a few particles containing desoxyribose nucleotides. The cytoplasms are poor in nucleic acids, with the exception of the cells in the *stratum granulosum*, in whose cytoplasm considerable quantities of ribose nucleic acids have been detected (Caspersson and Nyström, 1941). These take part in the protein generation during the horn formation in the skin. Thanks to the characteristic structure of the Molluscum colonies, it is possible by investigating different layers to obtain an impression of the sequence of the different processes. Fig. 1b-e are part of a series of photographs taken from outside towards the center of the mollusc. Fig. 1b is of the *stratum spinosum*. A comparison with normal skin shows that the cells have been greatly enlarged. The protein concentrations

are the same as in normal cells when measured by spectrum analysis, implying that the quantity of cytoplasm protein in each cell has greatly increased. This augmentation is effected by the nucleus of the cell, which shows all signs of intensive activity.

After the cell has attained a certain size (Fig. 1c) there appears in the cytoplasm, at first faintly and then with progressive distinctness, a network of absorbing material, which rapidly fills the entire cytoplasm (Fig. 1d). The nuclei are displaced to the peripheries of the cells and are flattened. Deeper in the tissue only large, ellipsoidal or irregular, strongly absorbing massive structures are to be seen (Fig. 1e). These are the Molluscum bodies. The finely netted structure contains large quantities of nucleic acids, and the large bodies into which these networks are transformed contain these substances in very high concentrations. The Feulgen nucleal reaction shows that the nucleic acids consist of desoxyribose nucleotides, *i.e.,* those which normally never occur outside the nucleus.

The cytochemical changes occurring on infection with Molluscum therefore appear generally to consist in a stimulating of the epithelial cells to extremely intensive production of protein. The material resulting from this process is transformed into masses of high desoxyribose nucleotide content, which are finally accumulated into the Molluscum bodies, while the other cell residues die.

2. *Verruca vulgaris* may serve as an example of a highly organized, desoxyribose nucleotide-containing virus, where the principal development occurs in the nucleus of the host cell.

As in *Molluscum contagiosum,* the chief features of the process appear when investigating the different cell layers. The first changes observed are the same nuclear alterations as occur in Molluscum, *i.e.,* signs of intensive functioning of the nucleoprotein-forming parts of the nucleus (Fig. 2a). It is a striking feature, however, that the increase in the cytoplasm protein does not exactly correspond to the cytochemical picture of the nuclei. Simultaneously, substances appear in the nucleus, around the nucleolus, which at first resemble the nucleolus-associated chromatin, but which soon increase at a rapid rate and ultimately fill the greater part of the nucleus (Fig. 2b-c). Finally they join together, forming aggregates similar to the Molluscum bodies, while the remainder of the cells die (Fig. 2e). As distinct from the case of Molluscum, the process is intranuclear throughout.

The cytochemical changes occurring in cells infected by the Molluscum and Verruca viruses may be summarized as follows:

In the course of the infection process, there arise in the cells in both cases large quantities of desoxyribose nucleotide-containing substances of a type not occurring in normally functioning cells. These substances combine to form large structures, corresponding to the large cell inclusions known from previous experience. These are characteristic for the infection in question. We may assume that the desoxyribose nucleotide-containing masses are identical with the virus masses, reproducing themselves under the influence of nucleotides. In the case of Molluscum they are localized in the cytoplasm, in that of Verruca in the nucleus.

It is striking how both infections stimulate the protein formation of the host cells. This occurs in Molluscum in the earliest stages of the infection, and a giant cell results. Thus the first stage in the infection consists in a protein formation. When the cell has attained a certain size the pathological substances appear, in the form of desoxyribose nucleotides, in the cytoplasm. This leads to the rapid apparent transformation of the large cell body into virus material. In the case of Verruca, the nucleus of the cell also shows signs of excessive stimulation. There is not much formation of cytoplasmic protein, however. Instead, around the nucleolus there appear masses, which are regarded as virus in a state of reproduction. Thus, in both cases analyzed, the cells are primarily stimulated to intensive protein production, but the products of this process are consumed by the reproduction process of the virus itself, which is also carried on with the aid of nucleotides. The cytochemical analysis thus demonstrates that the virus exploits the nucleoprotein-forming parts of the host cell in order to obtain raw material for its own growth. In the first case it acts parasitically on the cytoplasmic level of the normal protein-forming apparatus, while in the latter case it immediately attacks the nucleolar level.

II. *Examples of neurotropic viruses reproducing themselves in the nucleus of the host cells*

The organization of the virus substance and its relation to the host cell in the course of reproduction can be studied particularly well in nerve cells infected with neurotropic viruses. Before discussing the experiments with neurotropic viruses, a survey will be given of the results obtained on the nerve cells under different functional conditions. The nerve cell generally lends itself well to the study of chemical intracellular processes, because of its large size and the possibility of defining the functional state in individual cells. The nerve cells occupy such a special position among the somatic cells of the organism, that it may be opportune here to review their cytochemistry during development.

Development of the nerve cell

Mitosis in the nervous system ceases at an early stage of the nerve-cell development. During the following stage the young nerve cell develops a large nucleolus and a cytoplasm equipped with several cytoplasmic processes. The total volume of the processes, exclusive of the myelin sheath, exceeds that of the cytoplasm many times. In an anterior

horn cell, the volume of the axon will exceed that of the cytoplasm about 1,000 times.

It is evident that the demands on protein production for the building up of this structure during embryological development must be very high. In the first stage of development the nerve cells correspond closely to other somatic cells, but after the stage of late unipolar neuroblasts they pass through a second period of growth. Computations based on data from ultraviolet absorption spectra show that the total amount of proteins in the nerve-cell cytoplasm increases more than 2,000 times during development. The cell volumes were computed as prolonged rotation ellipsoids, and the amounts of nucleic acids were determined both by including and by excluding the volume of the cell nucleus. If the axon is included in the calculation the values are increased to between 30,000 and 200,000 times, depending upon the length of the axon (Hydén, 1943a). It is not surprising, in view of the other data obtained in experiments on different materials, that the nerve cells during development produce a large nucleolus rich in ribose nucleoproteins as well as large amounts of ribose nucleoproteins in the cytoplasm. The nerve cell is equipped, from the very beginning, for intense protein production. Furthermore, this capacity persists even in the adult stage—a feature which is unique among the tissues of the mammalian organism.

This was quite astonishing; however, further studies showed that the cytoplasmic nucleic acids in the nerve cells are strongly correlated with functional activity. During the function of the neuron, large and rapid changes occur in the content of nucleotides and proteins of the cytoplasm.

The composition of certain parts within the nerve cell nucleus

In view of the importance, in cellular production within the cell, of the heterochromatin and of its derivative, the nucleolus-associated chromatin, and the significance of this cell organelle in nerve cells (Hydén, 1943a, b; Hamberger and Hydén, 1945), a brief description will be given of the organization and composition of the nucleolus-associated chromatin in the nerve cell.

Near the nucleolus of the nerve cell is a region having a higher absorption at 2600 A than is characteristic of the remainder of the nuclear substance (Fig. 3). This region within the nucleus contains proteins rich in basic groups and has, moreover, small concentrations of ribose nucleotides with small particles rich in desoxyribose nucleic acids interspersed. This area of high protein concentration belongs to the nucleolus-associated chromatin.

Thus the nucleolus-associated chromatin in the nerve cell is separated into two parts: (1) the above-mentioned area in the vicinity of the nucleolus (Fig. 3), and (2) the part immediately surrounding the nucleolus and containing desoxyribose nucleotides (Fig. 11a).

The hyperfunction of the nucleolus in the nerve cells

In spinal ganglion cells of certain fishes—*Lophius piscatorius*, Gadus and Esox—an intense activity of the nucleolus and the nucleic acid forming parts of the cell can be shown (Hydén, 1943b).

In the main part of these cells it is possible to observe by direct measurements the formation of an accumulation of ribose nucleic acids in the cytoplasm at the outer side of the nuclear membrane (Fig. 4). This occurs within a well-defined area of the nuclear membrane, which is irregularly folded within this section. The mechanism is the following. In the nucleus a gradient in the concentration of the protein from the nucleolus toward the nuclear membrane within the folded area can be observed, indicating a migration of protein substances. At the outer side of the nuclear membrane ribose nucleotides are concurrently formed and the cytoplasmic proteins increase.

This hypertrophy of the nucleolus in the nerve cells works in many cases as a response to increasing functional demands. It can be shown in experiments with sensory and electrical stimulation, with acoustic stimulation, in the recovery period after sectioning of the axon, and under physiological conditions in the Purkinje cells of the cerebellum (Hydén, 1943; Hamberger and Hydén, 1945).

Motor stimulation of anterior horn cells

In the course of intense muscular work, with increased neural function, there occurs a sharp decrease in the content of protein and nucleic acids in the cytoplasm of the anterior horn cells. The difference in the protein and nucleotide content of the nerve cells between animals at rest and in a state of exhaustion is considerable, amounting to between 3 and 5 times (Hydén, 1943b).

These experiments show that during intense muscular work, with increased function of the respective motor nerve cells, diminution in the content of nucleoproteins occurs. The original values are restored during the first 48 hours after exercise. This signifies that the function of the nerve cell is accompanied by metabolism processes involving extensive quantitative changes in the nucleoprotein content.

Sensory stimulation of nerve cells

As an example, I will mention an experiment performed on 150 guinea pigs, employing acoustic stimulation and subsequent investigation of the cytochemical changes in the nerve cells of the cochlear ganglion (Hamberger and Hydén, 1945). One advantage of these nerve cells is their uniformity of cytochemical composition under acoustically quiet conditions. On acoustic stimulation with tones of various frequencies and intensities, the nerve cells of the cochlear ganglion undergo extensive cytochemical changes. On exposure, for example, to a tone of a frequency of 6,000 cyc/sec

an intensity of 80 db. for 3 hours, the ganglion cells pass through a cycle of changes lasting 3 weeks. Following the stimulation, the cellular protein and nucleotides diminish, the diminution being most marked during the second week. The protein concentration diminished from 30-35% to 2-10% and the nucleic acid concentration decreased from values around 2% to <0.1%.

In the third week the original protein and nucleotide concentration is restored. The first sign of this phase is an accumulation in the cytoplasm around the nuclear membrane of ribose nucleotides and proteins.

Acoustic trauma caused by reports from a revolver likewise entails diminution of the cellular protein and nucleic acid content. In that case, however, there are signs of damage to the cells. The diminution is not completely restored even after a lapse of 8 weeks after the experiment, although most of the cells by that time show signs of restoration.

The results of the experiments with acoustic stimulation also lead to the conclusion that the changes in the nucleoprotein content in nerve cells are normal processes correlated with function.

The connection between nucleoprotein metabolism in the nerve cells and psychic function

From both the theoretical and the practical point of view it should be of interest to investigate whether it is possible to influence the content of nucleotides and proteins in nerve cells by means of chemical substances. A suitable substance was found in malononitrile, $CH_2(CN)_2$. It was found in experiments started in 1944 (Hydén and Reuterskiöld, 1947) that after this substance was administered to animals a considerable change occurred in the cytochemical composition of the nerve cells in the central nervous system.

If malononitrile is administered to animals in sufficient doses, about 4 mg/kg of body weight, a great increase in the content of protein and nucleic acids in the nerve cells can be observed. This effect is noticeable in both cytoplasm and nucleus 1 hour after intravenous injection. The amount of nucleoproteins in the cytoplasm of motor cells increased 2-3 times after treatment.

Figs. 5 and 6 give examples of two anterior horn cells from a rabbit treated with malononitrile. Fig. 7 (upper curves) gives examples of some absorption spectra taken at points in the cytoplasm of an anterior horn cell from a rabbit treated with malononitrile. It was not possible to produce this effect on cells

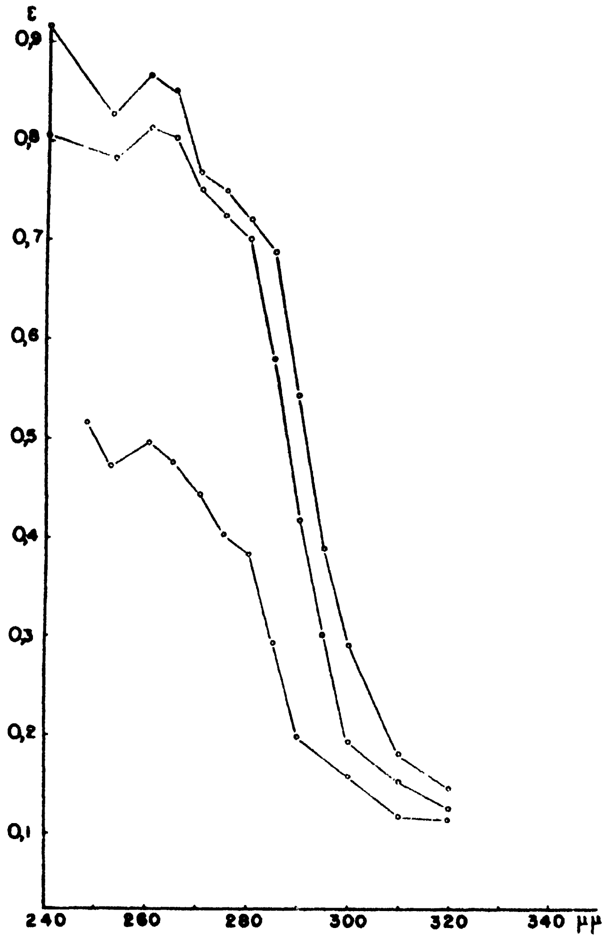


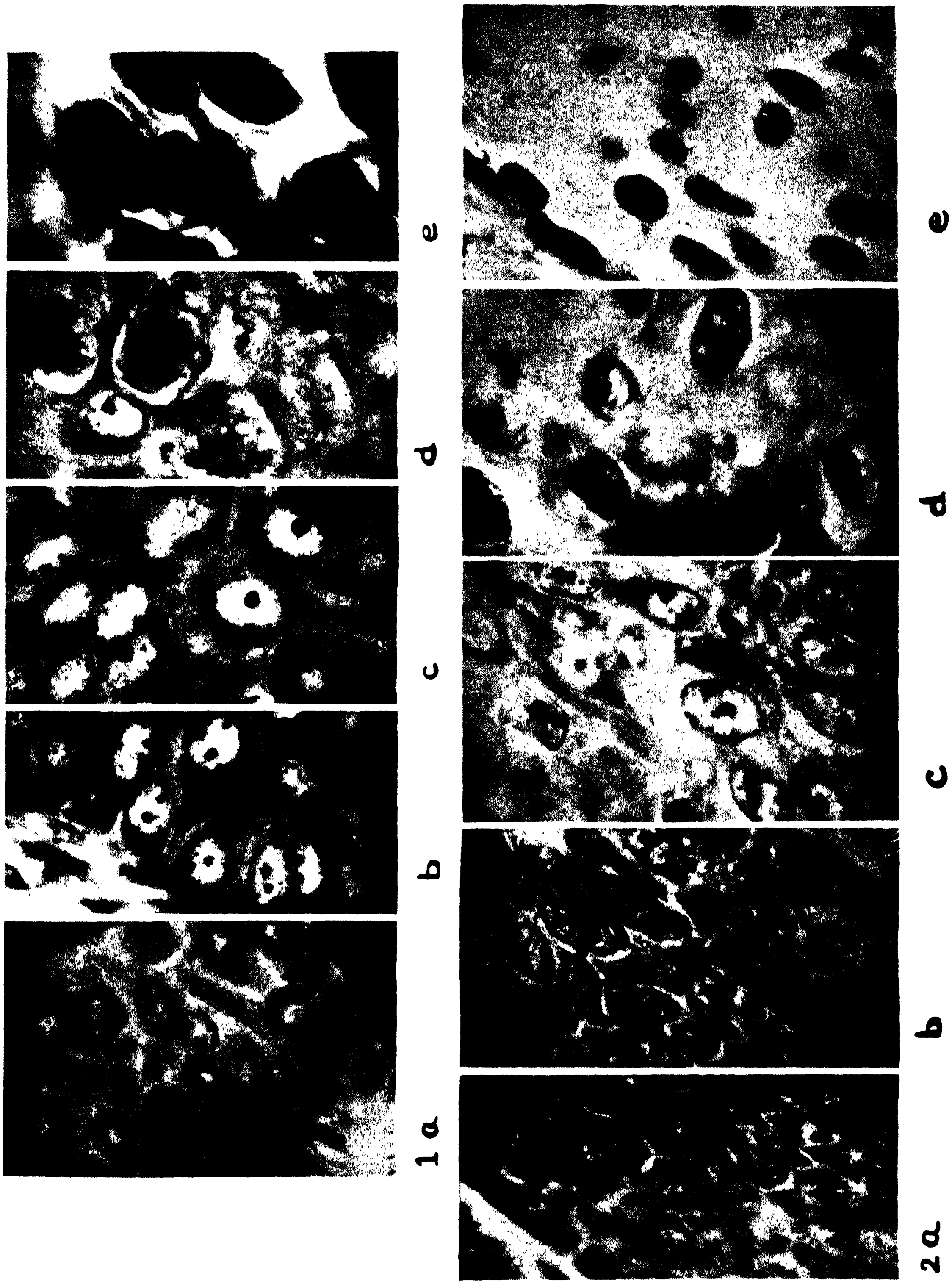
FIG. 7. Absorption spectra from points in the cytoplasm of the cells in Figs. 5 and 6 (2 upper curves). High maxima at 2600 A and also at 2800-2900 A. Below is drawn an absorption spectrum from the cytoplasm of a corresponding anterior horn cell from a control animal. All cells treated and fixed in the same way.

LEGENDS FOR FIGURES 1 AND 2 (see opposite page)

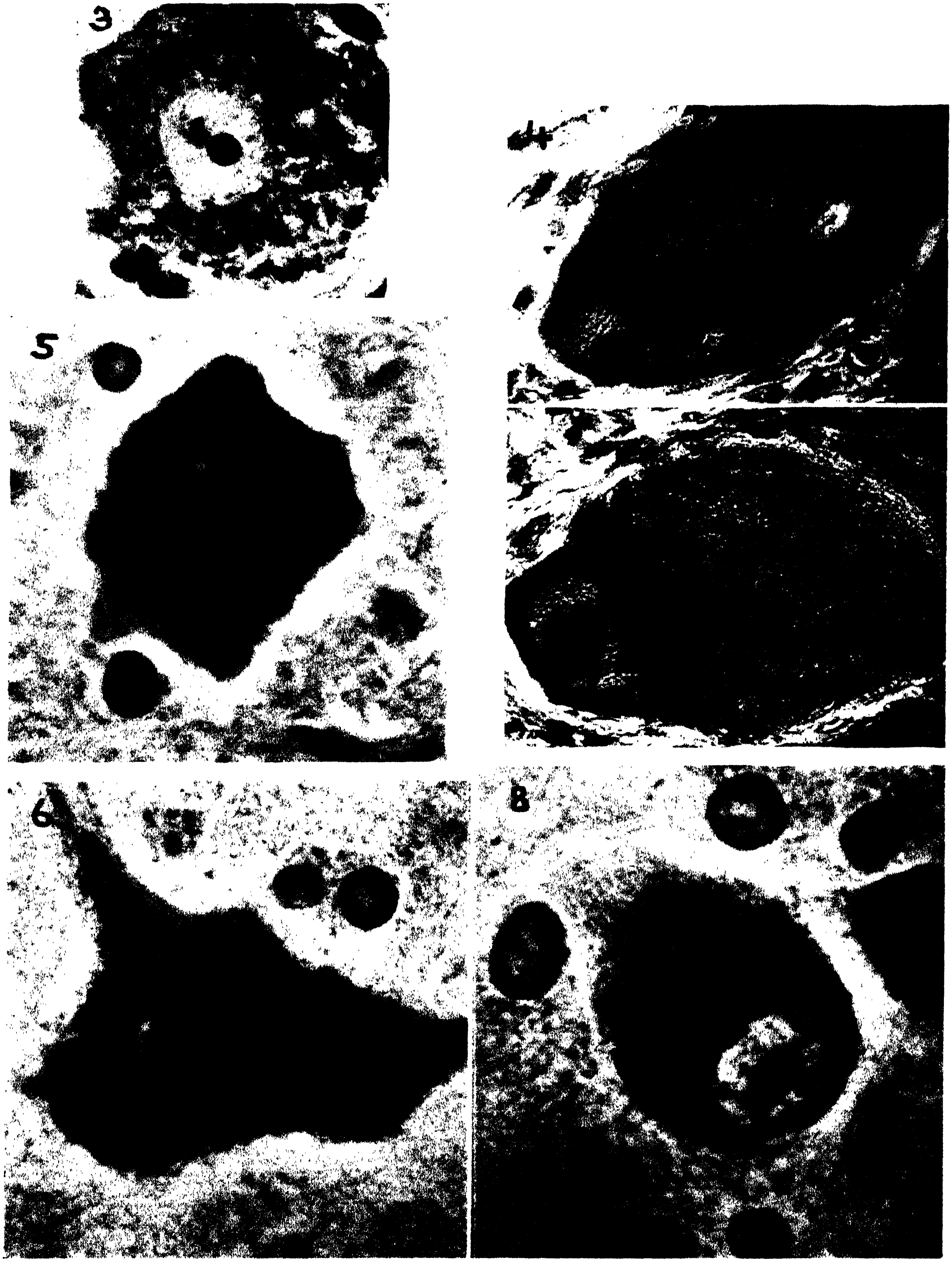
FIG. 1. A series of pictures in ultraviolet at 2570 A taken from the surface towards the center of a Molluscum tumor. The photographs give a survey of the development of the nucleoprotein-containing Molluscum bodies.

FIG. 1*a* shows a section of the stratum spinosum of normal skin. FIG. 1*b* shows the corresponding layer in the Molluscum tumor. The cell volumes have increased and large nucleoli have developed. FIG. 1*c-d* shows the occurrence of an absorbing network in the cytoplasm. It increases in the cells nearer the center (1*d*) and the nuclei are pushed aside and destroyed. In FIG. 1*e* the fully developed Molluscum bodies containing nucleoproteins in high amounts. Magnification 200 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 2*a-d*. A series of ultraviolet pictures taken from a common wart, *Verruca vulgaris*, from the surface towards the center. The pictures show the development of the nucleoprotein-rich Verruca bodies (FIG. 2*d*), the generation taking place in the nucleus from the site of the nucleoli (FIG. 2*a-c*). Magnification 2000 ×. Objective aperture 0.85. Condenser aperture 0.6.



FIGS. 1 and 2 (see opposite page for legends).



FIGS. 3, 4, 5, 6 and 8 (see opposite page for legends).

of other tissues than the nervous system. Control experiments were carried out on pancreas and glandular cells. Control experiments and calculations showed moreover that the effect is not due to a fixation of the $CH_2(CN)_2$ groups to the nerve cells, but to a real increase of the polynucleotides and protein.

Experiments on the mechanism by which the malononitrile may increase the nucleoproteins of the nerve cells have been started and some preliminary results will be mentioned.

Malononitrile seems to inhibit strongly the cyanide sensitive enzyme systems. In some experiments with catalase carried out according to Bonnichsen (1947), the catalase effect was inhibited as much as 30-35% by 0.007 M malononitrile.

Malononitrile seems to influence the nucleic acid content in the nerve cell by means of inhibiting cyanide sensitive enzyme systems. This conception is supported by cytochemical experiments on the effect of ischemia and insulin on the nervous system, which are to be published elsewhere. Ischemia of short duration and small doses of insulin also increase the content of nucleoproteins in motor nerve cells, though not to the same degree that malononitrile does (Hochberg and Hydén, 1947). The inhibitory effect on the cells produced by ischemia and insulin might in principle be considered to be the same as that of malononitrile on the cyanide-sensitive enzyme systems.

These animal experiments furnished a basis for demonstrating the connection between nucleoprotein metabolism and function in the nerve cells on quite different biological material, namely, on human beings.

The reported experiments on animals showed that protein and nucleic acid changes in the nerve cells are associated with their function. It was therefore thought possible that stimulation of nucleoprotein production in the nerve cells would likewise entail stimulation in function in those cases where the nucleoprotein production was low. In cases of manic-depression and schizophrenia of long duration it was found that the content of protein and nucleotides in various kinds of nerve cells was low. This was especially the case in manic-depression. The material was collected from both healthy and diseased subjects, partly in connection with leucotomia operations and partly at post-mortem within 3 hours after death.

Treatment with malononitrile has been carried out on patients at Beckomberga sjukhus (Hydén and Reuterskiöld, in press) and at the Psychiatric Clinic of the Karolinska Sjukhuset, Stockholm (Hartelius and Hydén, 1947). Altogether about 130 patients have been treated, up to 10 times each. They have been given malononitrile in doses from 3-5 mg/kg of body weight intravenously, and the substance has been permitted to work for one hour. It was found that the effect on endogenic depression was most favorable. In most of these cases the inhibitions disappeared gradually in the course of the treatment or immediately after it. In most of the cases of endogenic depression the symptoms of the illness gradually disappeared during treatment with 2 or 3 injections per week. The more pyknic the patient was in type, the better malononitrile was found to act. Cases have been followed for as long as 18 months.

Even in cases of schizophrenia of long duration some of the catatonic symptoms disappeared and the patients showed evidence of mental stimulation. The experiments with man started with cases of schizophrenia of long duration and the effect on this disadvantageous material was noticeable in 48 hours after treatment and remained up to one week.

The experiments with malononitrile in the treatment of psychotics are proceeding, and in this brief report I wish merely to stress the main point in these experiments from the physiological point of view: The correlation between nucleic acids and protein changes and function in the nerve cell, which was shown in animal experiments, seems to be confirmed in regard to psychic function in man.

In summarizing the most important experiments on nerve cells, which form a background for further studies on neurotropic viruses, the following facts emerge. Extensive quantitive changes in the nucleic-acid and protein content of the nerve cells are correlated with motor and sensory function. This correlation seems also to be valid in relation to psychic function.

LEGENDS FOR FIGURES 3, 4, 5, 6 AND 8 (see opposite page)

FIG. 3. A spinal ganglion cell from a rabbit photographed at 2750 A. Near the nucleolus the absorbing chromocentral area is rich in protein of a basic character. Magnification 1000 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 4. Spinal ganglion cells from *Lophius piscatorius* taken at 2570 A. Note the large nucleoli and the nucleotides at the folded nuclear membrane. The cells are in the stage of protein formation. Magnification 750 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIGS. 5 and 6. Anterior horn cells from the antero-lateral group of the spinal cord of a rabbit. The cells are photographed at 2570 A. 2 hours after the injection of 4 mg $CH_2(CN)_2$ per kg body weight. Note the strong absorption in both nuclei and cytoplasm. Magnification 2000 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 8. Purkinje cell from a rabbit photographed at 2570 A. Note the high absorption around the nuclear membrane which is due to ribose nucleotides. This cell represents one of the stages in the nucleic acid metabolism in the Purkinje cells and is characterized by a small content of nucleic acids in the cytoplasm. Magnification 2000 ×, × 2. Objective aperture 0.85. Condenser aperture 0.6.

Examples of neurotropic viruses containing desoxyribose nucleotides

1. *Rabies.* Both viruses of "type fixe" as well as that of "Street" type including the "Tanger" strain of rabies have been used. The latter was kindly provided by Professor Levaditi of Paris and by Dr. D. Harris of St. Louis. The experiments were carried out on rabbits and on dogs. The material was taken at different times after intracerebral infection with the virus, starting during the incubation period. A special study was made on the Purkinje cells, since for several reasons these constitute an excellent subject. The very extensive changes in nucleoprotein content that occur in these cells under physiological conditions have previously been the subject of a thorough analysis (Hydén, 1943a).

Even in a simple photographic study made in the ultraviolet range, one is struck by the great differences in absorbing power between the different cells. Spectral analysis shows that these differences, conditioned by the functional state, are due to the fact that the content of nucleic acids and protein is small or moderate in some cells and very high in others. Fig. 8 shows a Purkinje cell of "type I," which contains nuclear-membrane nucleotides but has otherwise only a moderate content of cytoplasmic protein and nucleotides.

When Purkinje cells are infected with rabies either of the "fixe" or the "Street" type, the majority show differences from the normal cytochemical picture even at an early stage of the infection. The nucleus will be filled by large quantities of foreign substances containing nucleotides and proteins in high amounts (Fig. 9). Fig. 10 shows examples of absorption spectra obtained from those newly formed substances. The lower curve is from an early stage and the upper curve from a later stage in the infection. An absorption maximum is seen at 2600 A, and another at 2750 A. A comparison between the proportions of nucleic acids and proteins shows that the component nucleotides occur in small amounts compared with the protein substances. The Feulgen reaction shows these nucleotides to be of the desoxyribose type.

Cytochemical analysis of such cells invariably shows the cytoplasm to contain only very small concentrations of protein. This indicates that the normal protein-generating processes in the Purkinje cell have ceased.

This material is extremely well suited for following the development of foreign nucleic acids and proteins in the nuclei of the Purkinje cells. In a normal Purkinje cell the Feulgen-negative nucleolus is surrounded by only a small number of particles rich in desoxyribose nucleotides, constituting the nucleolus-associated chromatin (Fig. 11a). The remainder of the nuclear substance contains only very small numbers of such particles. In the infected cells the growth of new substances begins at the site of the nucleolus-associated chromatin (Fig. 11b).

FIG. 10. Absorption spectra taken at points in the substances generated in the nuclei of Purkinje cells infected with rabies. Curve below taken from the cell in Fig. 9*a*, upper curve from the cell in FIG. 9*d*. Strong absorption maxima at 2600 A as well as at 2800 A. The height of the maximum at 2600 A in the upper curve indicates greater amounts of polynucleotides in that type of cell compared with the cell in Fig. 9*a*, the latter representing an earlier stage in the infection.

Somewhat later, the nucleus is filled by a large number of particles (Fig. 11c-d), and ultimately all the normal nuclear details disappear (Fig. 9d). The cytoplasm as a whole has a low absorption power in the ultraviolet range, since it contains proteins only in low concentrations and no nucleic acids.

2. *Neurovaccinia.* Although not a neurotropic virus in a strict sense, neurovaccinia is very well suited for cytochemical studies of the processes following intracerebral infection. The symptoms following infection develop during a very limited period of about 4-5 hours on the third day after intracerebral infection in rabbits, concurrently with the symptoms of paresis. A process similiar to that

observed in rabies-infected cells can be seen to develop during this very brief period. The normal nucleotide and protein metabolism in the cytoplasm in the Purkinje cells ceases, but in the nucleus are developed large amounts of substances containing desoxyribose nucleic acids and proteins. (Figs. 12 and 13). In a spectrographic study of suspensions of the corpuscles of neurovaccinia, Giuntini (1941) found a weak absorption maximum around 2800 A.

Examples of neurotropic viruses containing ribose nucleotides

1. *Poliomyelitis.* In infection of the motor nerve cells with poliomyelitis virus, the conditions seem to be more complicated. Preliminary experiments were therefore made in order to investigate the general character of the changes that occur.

Experiments have been carried out on monkeys and on mice, in the latter case using the Armstrong strain. Human material has been collected from 30 selected cases in the 1944 to 1947 poliomyelitis epidemics in Stockholm.

Only those cases were selected which ended in death after a few days of total paresis. Although the changes in nerve cells infected with poliomyelitis seem to vary at the first sight, it is possible by following cases which live about equally long after the infection to find corresponding stages in the course of the infection.

The first dectable changes involve the nucleus. In this first stage the infection brings about a stimulation of the nucleolus which corresponds in principle to the first stage of the Molluscum infection during which the cell protein and the nucleic acid content of the nucleolus increases.

Microspectrographic measurements show a rapid increase in the ribose nucleotide content of the nerve cell nucleolus in comparison with that in corresponding cells from healthy control material. Fig. 14a shows an uninfected anterior horn cell photographed with ultraviolet. Fig 14b shows a corresponding cell in this early stage of infection with poliomyelitis. Both were taken at 2570 A under identical conditions. The immense size of the nucleolus is especially impressive. The amounts of nucleotides and protein in these nucleoli have increased greatly.

In some early cases anterior horn cells of a certain cytochemical composition were observed in certain areas of the spinal cord; an ultraviolet picture of this is shown in Fig. 14d. Most characteristic are the large nucleolus and the ribose nucleic acids on the outside of the nuclear membranes in a well-defined area. This cytochemical picture is characteristic of nerve cells in which there occurs a rapid cytoplasmic protein production; for example, during the development of the neuron, in the recovery period after acoustic stimulation, and during the outgrowth period after sectioning of the axon.

Thus this first period of poliomyelitis infection consists in a stimulation of the nucleolus in the nerve cell.

Succeeding that period, there is a stage in which the nucleus is filled with substances not regularly occurring in the healthy nerve cell. In the ultraviolet picture the substances are seen to consist of small, more-or-less confluent granules which absorb fairly well (Fig. 14c). Absorption spectra (Fig. 15, curve 1) show that they contain proteins and nucleotides. The nucleotides are of the ribose type. The composition of those granules differs, however, from that characteristic for the protein-rich area in the vicinity of the nucleolus, in that they contain more ribose nucleotides.

In the next phase the nucleolus shows signs of severe damage, with a loss of nucleoproteins (Fig. 14e). At the same time the nucleotides of the cytoplasm disappear and the protein greatly diminishes. In the nucleus, however, the masses of foreign substances have retained their content of nucleic acids and protein; but they have changed their form and apparently contracted, and absorption spectra show that their content of nucleic acids has increased (Fig. 15, curve 2).

Hence in poliomyelitis infection also a disturbance in the content of nucleoproteins of the nerve cell can be established. I wish to emphasize that the cytoplasm is depleted of nucleoproteins which indicates that the function of the neuron has ceased. This disappearance of the nucleoproteins of the cytoplasm is correlated with the production of nucleoproteins in the nucleus which do not exist in the normal nerve cell.

This disturbance affects an early stage of the protein production of the cell, earlier than is the case in rabies, and apparently attacks the heterochromatic parts of the cell.

2. *Louping ill.* This infection was studied in mice and rabbits. Cytochemical changes following the infection were especially significant in the large motor cells and in the Purkinje cells of the cerebellum. Fig. 16 shows a large motor cell from the medulla oblongata at the culmination of the symptoms following intracerebral infection with louping ill. In this infection, too, nucleoproteins which do not occur in normal nerve cells are produced in the nucleus. The nucleic acids are of the ribose type.

The course of the nucleoprotein formation corresponds essentially to that occurring in poliomyelitis. Example of a spectrum obtained from the absorbing material in the nucleus of the nerve cell of Fig. 16 is shown in Fig. 17. All spectra obtained have shown that the amount of protein in the produced nucleoproteins is high in proportion to the nucleic acids. The cytoplasm absorbs very slightly, which is due to the minute amounts of proteins. Absorption spectra show that the nucleic acid content of the cytoplasm is practically negligible, the data obtained thus indicating a cessation of function of these cells.

Discussion

Summarizing the results from virus infected cells, the findings are in general strinkingly similar. Both in Molluscum and Verruca, as well as in rabies, poliomyelitis, and louping ill, pathological products appear in the cells. Like all self-producing systems previously investigated, they contain nucleic acids and protein groups.

In all probability these nucleoproteins produced in the cells during virus infection can be regarded as the viruses themselves in reproduction.

The newly generated desoxyribose nucleic acids in Molluscum, Verruca, rabies and neurovaccinia are in such amounts that never can be detected in the corresponding cells of control animals.

In the cases of cells infected by neurotropic viruses, I wish to emphasize especially one observation. The cytoplasm of the virus-infected cells is depleted of nucleoproteins. This observation cannot be compatible with the view that the virus is present in the cytoplasm. The present observations show on the contrary that the viruses reproduce themselves in the nucleus, though such a view may seem disagreeable to many a virus investigator.

There is, however, a striking difference between the types of nucleic acids found in the different cases. In Mollusca, Verruca, rabies, and neurovaccinia, *desoxyribose nucleic acids* and proteins were found. According to observations of the particle size of these viruses carried out by different methods (see Elford, 1938)—determination by filtration, ultraviolet microscopy, and electron microscopy—all of them are of a considerable size, ranging from 100 to 200 mμ.

In the cases of poliomyelitis and louping ill, on the other hand, *ribose nucleotides* are formed. Observations of the particle sizes of these viruses have shown that they range between 15 and 25 mμ.

In the table below are listed these data from which a correlation between the type of nucleic acids and virus particle size is evident. This permits a division of the investigated viruses into two groups.

Group I. Virus	*Particle size*	*Virus nucleic acids observed in the infected cells*
Molluscum	180-200 mμ	Desoxyribose nucleic acids
Verruca	150-200 mμ	Desoxyribose nucleic acids
Rabies	150-200 mμ	Desoxyribose nucleic acids
Neurovaccinia	150-200 mμ	Desoxyribose nucleic acids
Group II		
Poliomyelitis	15-25 mμ	Ribose nucleic acids
Louping ill	15-25 mμ	Ribose nucleic acids

The cytochemical observations, correlated with the observations of particle size and macrochemical observations, may serve as a basis for discussing the organization of the virus.

Virus Organization

In poliomyelitis and louping ill, ribose nucleotides are formed. Macrochemical investigations have shown the same condition in infections with plant viruses which are built up of a protein with associated ribose nucleic acid groups.

The *ribose nucleotides* consequently appear to characterize the virus species having the lowest degree of organization.

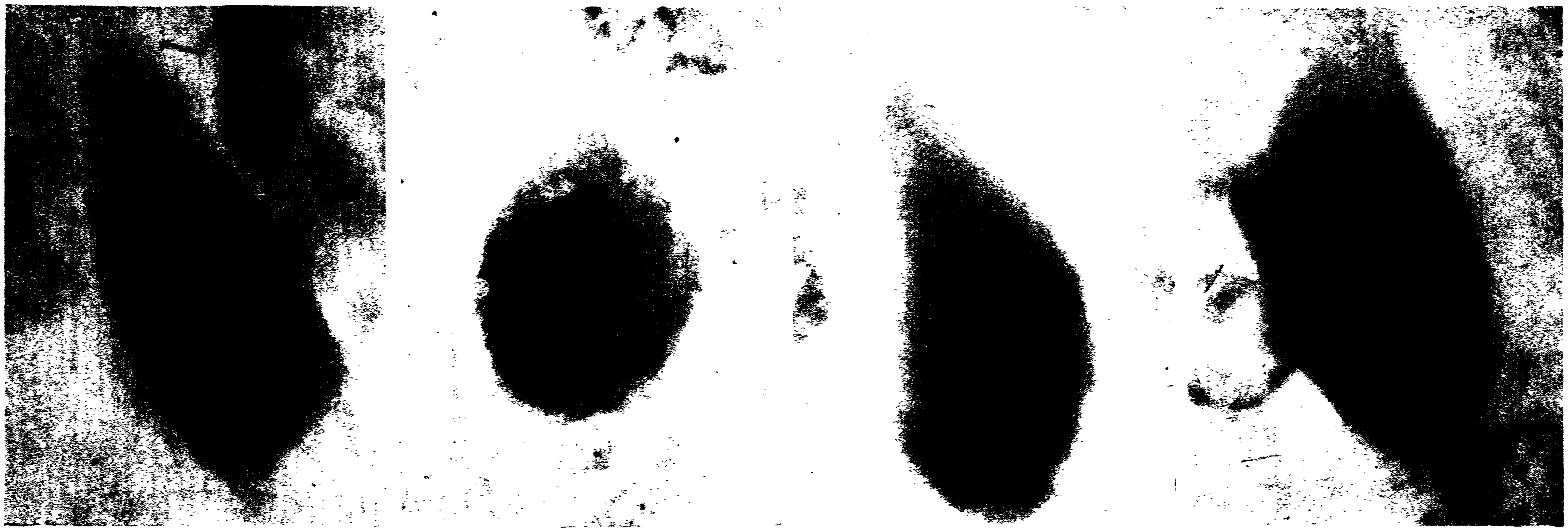
They are replaced by *desoxyribose nucleotides* as soon as the organism becomes complicated enough to contain several different protein groups which in reproduction must be equally divided among the daughter organisms. For this equal distribution to take place, a linear arrangement must be presumed to prevail during reproduction; that is, a chromosome must be formed.

Throughout the plant and animal kingdoms, whenever it has been possible to identify chromosomes cytologically, the desoxyribose nucleotides are firmly bound to these and are characteristic of them. This distinction is more clear in the compound nuclei of the more highly organized cells.

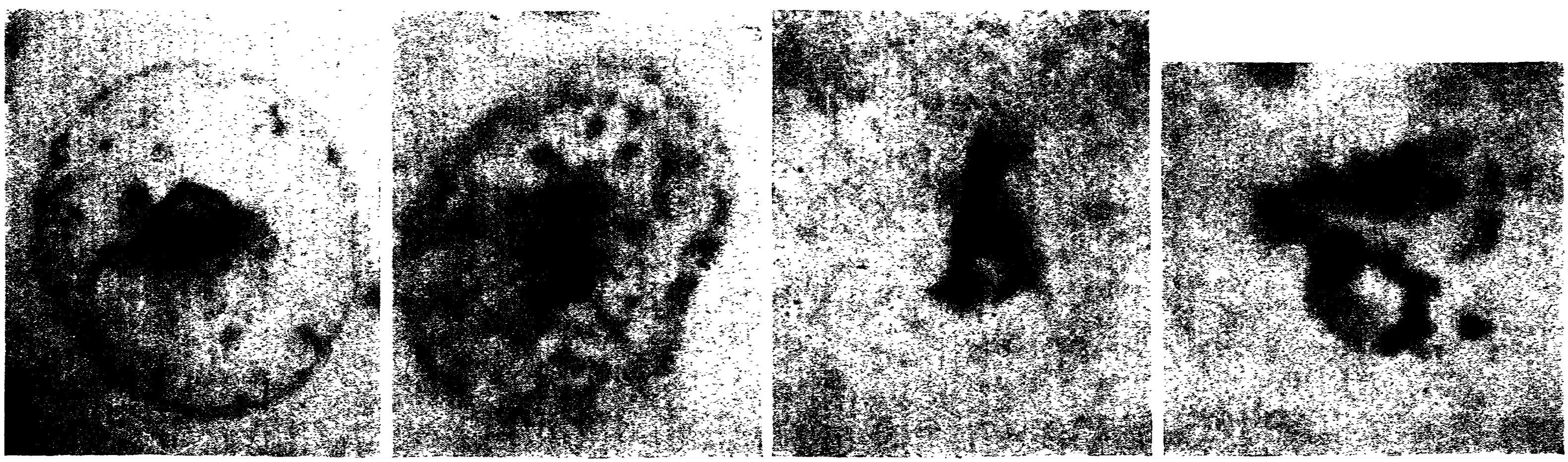
LEGENDS FOR FIGURES 9 AND 11 (see opposite page)

FIG. 9. Series of photographs at 2570 A of Purkinje cells from rabbits infected with rabies. The cells represent different stages in the infection. All the cytoplasms absorb poorly corresponding to a low content of protein and practically no nucleic acids. The ordinary picture of the nucleus has completely changed and the nucleus is filled by strongly absorbing substances, the development of which can be followed in *a-d*. In *d* the nucleus and also the major part of the cytoplasm seems to be completely filled. Magnification 2000 ×, × 2. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 11*a*. Feulgen's nucleal reaction carried out on a Purkinje cell of a control rabbit. Few particles rich in desoxyribose nucleic acids are seen in the nucleus, the major part of them collected around the nucleolus as the nucleolus-associated chromatin. In *b-d* the development of Feulgen positive nucleic acids in the nucleus during rabies infection can be followed, the absorption spectra of which are seen in FIG. 10. The production begins at the nucleolus-associated chromatin at the opposite poles of the nucleolus (FIG. 11*b*) where two strongly absorbing buds can be seen which give a positive Feulgen reaction. FIG. 11*c-d* represent later stages.



9a b c d



11a b c d

FIGS. 9 and 11 (see opposite page for legends).

LEGENDS FOR FIGURES 14 AND 16 (see other side)

FIG. 14*b–e*, A series of photographs taken at 2570 A of anterior horn cells from monkeys infected with poliomyelitis representing different stages in the infection. FIG. 14*a* represents a cell from the antero-lateral group of a control animal. Note the impressive nucleolus in FIG. 14*b* and the absorbing masses in the nucleus in FIG. 14*c*. In the cell in FIG. 14*d* nuclear membrane nucleotides are clearly visible. The cytoplasm of the cell in FIG. 14*e* absorbs faintly, corresponding to a minute content of proteins and practically no nucleic acids. In the nucleus are seen many small strongly absorbing particles. See text. Magnification 2000 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 16. Large motor nerve cell from medulla oblongata of a rabbit infected with louping ill. The cell is photographed at 2570 A at the culmination of the infection. The absorbing capacity of the cytoplasm is almost negligible. In the nucleus there are strongly absorbing masses. Magnification 2000 ×. Objective aperture 0.85. Condenser aperture 0.6.

FIG. 15. Absorption spectra of the substances developing in the nucleus of anterior horn cells during infection with poliomyelitis. Curve 1 from a cell as seen in Fig. 14*c*, curve 2 from a cell as seen in Fig. 14*e*. The absorption maximum at 2600 A in curve 2 is considerably higher than that in curve 1 indicating that apart from a structural contraction the content of nucleic acids also has increased. In both curves distinct absorption maxima at 2800-2900 A.

The ribose nucleic acids take part in the increase of the homogeneous protein masses, whereas the desoxyribose nucleic acids appear when chains of protein groups with wide varieties of links are to be reduplicated in the chromosome apparatus. Thus the nuclear organization, from the simplest virus to the higher animals and plants, seemed to be based upon the same fundamental principle (Caspersson and Hydén, 1945).

THE RELATIONSHIP BETWEEN THE VIRUS AND THE HOST CELL

In rabies, neurovaccinia, and Verruca the similarity is so striking that the point of attack on the host cell is obviously the same in all three cases, *i.e.*, the nucleolar stage in normal nucleoprotein production, which is devoted to the formation of virus substance.

The poliomyelitis and louping-ill viruses appear

FIG. 17. Absorption spectra taken from the generated masses in the nucleus of the cell photographed in Fig. 16. The spectrum shows a strong maximum at 2750 and a smaller maximum at 2600 A indicating proteins in high amounts and nucleic acids in smaller amounts.

to make their attack at a yet earlier stage. The result is deleterious to the nucleoprotein forming parts and hence to the life of normal cells. Fig. 18 shows, in very schematic form, the points of attack for the different species of viruses. *Hence on the basis of the present data the viruses can be defined as self-reproducing units which act as parasites upon the nucleoprotein forming parts of the host cells.*

CONCLUSIONS

These investigations may be summarized as follows. A study has been made of a number of virus species with respect to their endocellular reproduction. The species were selected so as to represent a number of virus groups, differing in their cytological characteristics. The results show decisively that the principle of the indispensability of nucleic acids in biological protein synthesis, *i.e.*, in reproduction, is applicable to these viruses.

The reproduction of the simpler viruses is mediated by *ribose nucleotides*. The more complicated viruses, however, must be presumed to contain several different protein groups, which are to be

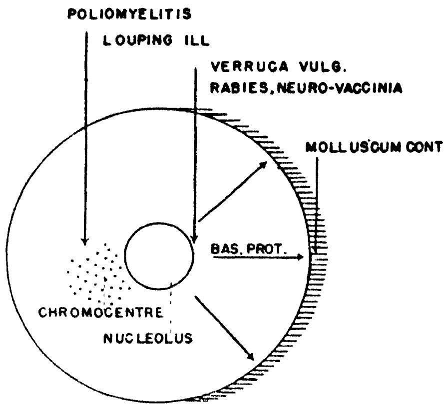


FIG. 18. Chart indicating the points of attack in the cell of the different viruses investigated. See text.

reproduced and divided among the daughter individuals. For this purpose there is established a primitive chromosome mechanism, containing *desoxyribose nucleotides,* which is comparable to the nucleus of more highly organized cells.

All the virus species investigated *behave as parasites on the nucleoprotein forming parts of the host cell.* The sites of their attack may be determined by means of the ultraviolet cytochemical technique, and are different for different species.

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NUCLEOPROTEINS AND VIRUS ACTIVITY

C. A. KNIGHT

Size and Shape and General Composition of Viruses

The viruses are infectious agents largely characterized by their small size and ability to reproduce only within the living cells of a particular host. While the particles of any given virus apparently possess a fairly definite and characteristic size and shape, the variation among different viruses in this respect is considerable. For example, the particles of tomato bushy stunt virus (Stanley, 1940) and those of the Lansing strain of poliomyelitis virus (Loring, Marton, and Schwerdt, 1946) appear to be essentially spherical with diameters of about 25 mμ; tobacco mosaic virus is represented by rod-like particles 15 × 280 mμ (Oster and Stanley, 1946); certain bacteriophages appear to have sperm-like forms (Luria, Delbrück, and Anderson, 1943; Hook, Beard, Taylor, Sharp, and Beard, 1946); and the elementary bodies of vaccinia are rectangular in shape with length and width approximately 260 × 210 mμ (Green, Anderson, and Smadel, 1942) (see Fig. 1). In general, viruses as a group appear to fill in the gap in size between the ordinary protein molecules of the chemist and recognized living organisms (Stanley and Knight, 1941). However, regardless of size and shape, all viruses share the distinctive capacity for multiplying only within living cells.

There seems to be, as in the case of size, a characteristic composition for a given virus, but there is considerable variation in this respect among different viruses. This is evident despite the fact that relatively very few of the many known viruses have been isolated and purified sufficiently to permit chemical analysis. If one considers the transformation substance of pneumococcus to possess virus activity, then the simplest virus known at present is a desoxyribonucleic acid (Stanley, 1938; Avery, MacLeod, and McCarty, 1944). Otherwise, the plant viruses are chemically the least complex, for all of those which have been highly purified have proved to be simple nucleoproteins (Stanley, Knight, and Demerre, 1945).

Closely resembling the plant viruses in general composition is the Shope papilloma virus which, however, apears to contain, in addition to protein and nucleic acid, about 1.5% of lipid (Taylor, Beard, Sharp, and Beard, 1942). Other animal viruses and a bacteriophage, as indicated in Table 1, also appear to contain lipid as well as nucleoprotein. Influenza virus has been found to contain, in addition to nucleoprotein and lipid, a polysaccharide composed of mannose, galactose and glucosamine units (Knight, 1947a); and vaccinia virus has been reported to possess small but possibly integral amounts of copper, flavin, and biotin (Smadel and Hoagland, 1942). There is a variation among the viruses which contain lipid with respect to both amount and kind of this substance. For example, the T2 bacteriophage is said to contain only about 2% of lipid and this is in the form of neutral fat (Taylor, 1946), whereas approximately one-half of the virus of equine encephalomyelitis is lipid and this consists of phospholipid, cholesterol, and neutral fat (Beard, 1945). It seems at present, therefore, that viruses in general are composed of various combinations of nucleic acid, and protein,

Table 1. General Chemical Composition of Some Viruses

Virus	Composition	Reference
Tobacco mosaic; other plant viruses*	Nucleoprotein**	Stanley et al., 1945
Shope papilloma	Nucleoprotein, lipid (1.5%)***	Taylor et al., 1942
T2 bacteriophage	Nucleoprotein, lipid (ca 2%)	Taylor, 1946
Equine encephalomyelitis	Nucleoprotein, lipid (48%)	Beard, 1945
Newcastle disease	Nucleoprotein, lipid (27%)	Cunha et al., 1947
Influenza	Nucleoprotein, polysaccharide, lipid (23%)	Beard, 1945 Knight, 1947a
Vaccinia	Nucleoprotein, Cu, biotin, flavin, lipid (4%)	Smadel and Hoagland, 1942

* Tomato bushy stunt virus, tobacco ringspot virus, tobacco necrosis virus, alfalfa mosaic virus, potato-X virus (latent mosaic of potato), potato virus "Y," and southern bean mosaic virus.

** In the first 2 instances listed in this table it can be concluded with reasonable certainty that the nucleic acid is linked to protein to form nucleoprotein. In the remaining instances a similar combination is assumed largely by analogy, although there is also in most cases experimental evidence in support of this assumption.

*** The numbers in each instance refer to the amount of lipid found present.

and frequently lipid, with extra-nucleic acid carbohydrate and certain other substances appearing only in, as yet, special cases. With regard to the fundamental requirements for virus activity, nucleic acid and protein can be singled out from the various chemical components mentioned above, for these two substances combined to form specific nucleoproteins (exemplified by the plant viruses) appear to comprise the minimum chemical requirement for virus activity, and moreover, nucleic acid and protein constitute the only two components common to all viruses.

Nucleic Acids of Viruses

Both pentose- and desoxypentosenucleic acids have been found in viruses and while, as indicated in Table 2, only pentosenucleic acid has been observed thus far in plant viruses, either or both types have been reported present in animal viruses. The quantity of nucleic acid found in different viruses ranges from a low of about 4% in the virus of equine encephalomyelitis to 37 and 40%, respectively, in T2 bacteriophage and in tobacco ringspot virus (see Table 2). This considerable range in the quantity of nucleic acid in different viruses must have special significance, but too little is known at present of the specific function of the nucleic acid to permit a precise definition of its importance.

Table 2. Quantity and Type of Nucleic Acid of Some Highly Purified Virus Preparations

Virus	Pentose-nucleic Acid %	Desoxy-pentose-nucleic Acid %	Reference
Equine encephalomyelitis	4.4		Beard, 1945
Influenza	3*	2	Knight, 1947 a
Vaccinia		5.6	Smadel and Hoagland, 1942
Tobacco mosaic and strains	6		Stanley and Knight, 1941
Newcastle disease	1.1**		Cunha et al. 1947
Shope papilloma		9	Taylor et al., 1942
Alfalfa mosaic	15		Ross, 1941
Tomato bushy stunt	17		Stanley, 1940
Tobacco necrosis	18		Bawden, 1943
Southern bean mosaic	21***		Miller and Price, 1946
T2 bacteriophage		37****	Cohen, 1946
Tobacco ringspot	40		Stanley, 1939

* This value is considered better than that of 2.3% given by Knight (1947a), for it has since been discovered that the factor used in converting pentose values into pentosenucleic acid values was in error.

** Both pentosenucleic and desoxypentose nucleic acids are claimed to be present but the quantities of each have not yet been reported. Together they account for about 1.1% of the virus (non-liped P×11).

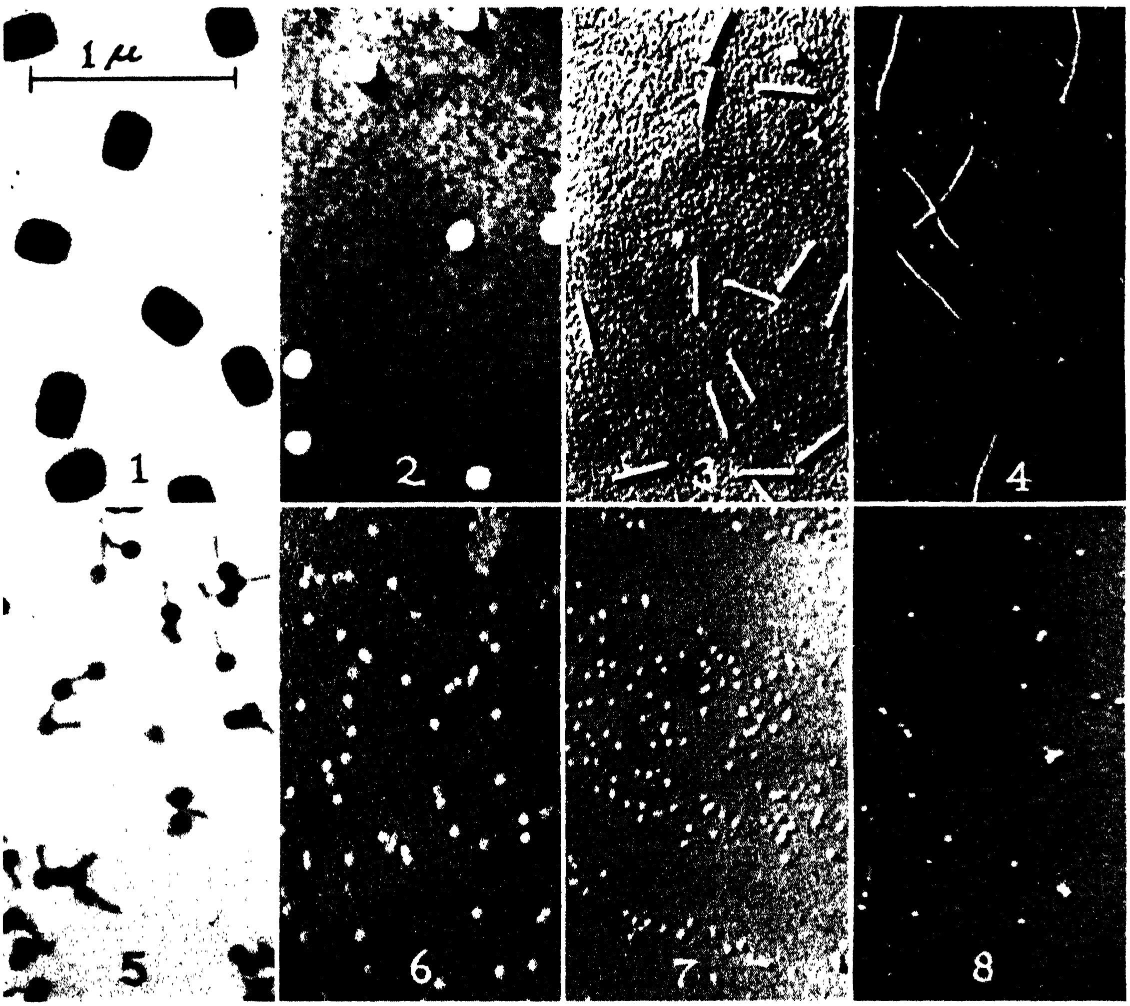
*** Phosphorus×11.

**** Taylor (1946) has reported the presence of both types of nucleic acid in T2 bacteriophage grown in broth or in a synthetic medium.

It is clearly evident from those viruses which consist solely, or almost solely, of nucleic acid and protein that these two components are linked to form a specific nucleoprotein compound. This conclusion is based not only on the constant proportion in which nucleic acid and protein are found in successive preparations of a given highly purified virus, but also on the observation that, in practically all instances, fairly vigorous treatment, such as subjection to strong alkali, is required to cleave the nucleic acid from the protein. In contrast it will be recalled that the nucleic acid can be separated from protein in the cases of a number of desoxypentosenucleoproteins simply by treatment with high concentrations of sodium chloride or by shaking with a chloroform-octyl alcohol mixture (Mirsky, 1943).

The nucleic acids removed from viruses have not as yet been found to possess any special properties, but it should be noted that in most cases the methods employed to release nucleic acid have been rather drastic and therefore the products obtained have usually been far from native. The work of Cohen and Stanley (1942) on tobacco mosaic virus furnishes a good example of how the properties of a nucleic acid can vary with the method of preparation. The pentosenucleate obtained by treatment of the virus with alkali was found to be only slightly viscous and to possess a molecular weight of about 15,000, whereas the pentosenucleate produced by heat denaturation of the virus under carefully controlled conditions was comparatively viscous, spontaneously birefringent, and had a molecular weight of approximately 300,000. The latter nucleate was considered to approximate in size the native nucleic acid of the virus and on the basis of this and some other assumptions it was possible to calculate that the virus possesses 8 threadlike nucleic acid units each having the length of the intact virus molecule.

The nucleic acid of tobacco mosaic virus was the first of these substances to be isolated from a virus and its chemistry has been rather thoroughly investigated. Loring (1939) found in early studies that this nucleic acid had the general properties and composition of a ribonucleic acid and analyses revealed it to be composed of the purines, guanine and adenine, the pyrimidines, cystosine and uracil, and a pentose, presumably ribose. From the hydrolytic products of the virus nucleic acid was isolated the brucine salt of uridylic acid which appeared to be isomeric rather than identical with the brucine salt of yeast uridylic acid. This indicated the presence in the virus nucleic acid of a new pyrimidine nucleotide. Continued work on this prob-



ELECTRON MICROGRAPHS OF SOME HIGHLY PURIFIED VIRUSES

FIG. 1. Vaccinia virus. FIG. 2. Influenza virus (Lee strain). FIG. 3. Tobacco mosaic virus. FIG. 4. Potato-X virus (latent mosaic of potato). FIG. 5. T2 bacteriophage. FIG. 6. Shope papilloma virus. FIG. 7. Southern bean mosaic virus. FIG. 8. Tomato bushy stunt virus. All mounts except 1 and 5 were shadowed with gold before examination in the electron microscope. The micrograph of vaccinia virus is a reproduction of that of Green, Anderson, and Smadel, 1942, and the remaining micrographs were obtained in this laboratory by Drs. Oster, Sigurgeirsson, Knight and Stanley.

lem, however, has shown the conclusion with respect to the uridylic acid to be in error and it has been concluded from a recent comparison of the physical and chemical properties of the nucleotides of yeast and virus nucleic acids that at least 3 of the corresponding nucleotides of the 2 nucleic acids, including uridylic acid, are identical (Schwerdt and Loring, 1947). It was not concluded, however, that the 2 nucleic acids have identical structures; on the contrary it was suggested that they did not. If this viewpoint is correct and if the findings with regard to pneumococcal transformation substances (McCarty and Avery, 1946) are considered to substantiate this belief, it would follow that in some as yet obscure way, the structures or molecular configurations, much more than the compositions, are responsible for the presumed biological specificities of different nucleic acids. Because of its potential significance in many fields of research, the relating of chemical structure to biological activity of any nucleic acid has become an important and challenging problem.

Virus Proteins

The viruses thus far examined have been found to consist of 50% or more of protein. In the case of the viruses of the tobacco mosaic group this value is about 94%. The protein contents of other viruses fall somewhere between the two figures just mentioned. Since the protein moiety constitutes such a large part of the substance of viruses, it seems reasonable to attribute many viral properties to this component. Therefore considerable interest has attended the chemical investigation of viral proteins.

It will be recalled that many nucleoproteins, particularly desoxypentosenucleoproteins, contain basic proteins of the protamine or histone type (Mirsky, 1943). In contrast, the protein of tobacco mosaic virus was very early recognized to be a comparatively acidic, globulin-like substance (Stanley, 1935). A chemical basis for this finding has subsequently been provided by the results of amino acid analyses which have revealed the presence of only about 11.5% of basic and about 25% of dicarboxylic amino acids (Knight, 1947b). Similarly the proteins of influenzal viruses have proved to be nonbasic (Knight, 1947c), as has also the protein of the Shope papilloma virus (Knight, 1947d). However, none of these viruses has the high nucleic acid content characteristic of sperm nucleoproteins. Perhaps analysis of the proteins of tobacco ringspot virus or of certain phages, which contain relatively large quantities of nucleic acid, will reveal basic proteins. However, for the most part, the protein and nucleic acid of tobacco ringspot virus are not separated by treatment with salt (Stanley, 1939), hence it seems unlikely that the protein of this virus resembles the protamines or histones. On the other hand, the proteins of viruses appear to resemble in a general manner those of some bacterial nucleoproteins, for the absence of salt dissociable linkages has also been observed with certain streptococcal nucleoproteins (Sevag and Smolens, 1941).

The chemically reactive groups of tobacco mosaic virus have been investigated with the object of discovering, if possible, which groups are essential for virus activity and also with the idea of producing known, and it was hoped, heritable changes in the virus molecules. Neither object was clearly gained but several interesting facts have emerged from the studies.

It has been found that some of each of the amino, phenolic, and indole groups of tobacco mosaic virus protein are probably part of the structure required for virus activity since the extensive reaction of these groups with formaldehyde (Ross and Stanley, 1938), 0.1 N iodine (Anson and Stanley, 1941), or with any of 5 more or less complex organic substituents resulted in loss of virus activity (Miller and Stanley, 1941; ibid 1942). Virus activity was partially restored in the case of the formolized virus by reversal of the inactivating reaction. The evidence at present does not, of course, exclude the possiblity that the observed inactivation of the virus in the several instances mentioned was the result of changes not revealed by the chemical tests applied. In this connection it should be noted that in certain cases it was found that 70% of the amino groups and 20% of the phenol plus indole groups could be substituted before measurable loss of biological activity was observed (Miller and Stanley, 1942). This can be interpreted as an indication that the integrity of only a portion of these groups is required for virus activity or it could mean that as yet obscure but vital changes in other parts of the virus were produced after the reaction had progressed to a certain level.

The oxidation of the cysteine sulfhydryl groups of the native virus to disulfide linkages resulted in no loss in virus activity (Anson and Stanley, 1941), which means either that the sulfhydryl groups are not essential for virus activity or that they are restored readily *in vivo* when the virus is tested.

As mentioned above in terms of percentages, several thousand organic substituents can be introduced into the tobacco mosaic virus molecule without causing appreciable loss of activity. However, multiplication of such virus derivatives or of those more completely substituted resulted in every case in the propagation only of the normal unsubstituted virus (Miller and Stanley, 1942). The conclusion which can be drawn at present from this data is that appreciable alteration of the chemically reactive groups of the virus does not affect the forces concerned in self-duplication of the virus. On the other hand, several of the substituents appeared to alter what may be termed the virulence of the virus, for

some of the virus derivatives were found to possess disproportionate activities when tested on two different hosts (Miller and Stanley, 1942).

The mutation of viruses is a phenomenon which in the form of its biological manifestations has been recognized for many years. In 1937 and later, it was shown that from plants diseased with strains of tobacco mosaic virus, crystallizable virus nucleoproteins could be obtained which possessed similar and yet distinctive physical and chemical properties (Stanley, 1937). Recently it has become possible to attempt a correlation of this knowledge with the fundamental chemistry of the viruses themselves. The first efforts in this direction took the form of chemical analyses on highly purified preparations of some strains of tobacco mosaic virus (Knight and Stanley, 1941). As described in a review of this work given at a symposium here six years ago (Stanley and Knight, 1941), qualitative tests showed that all of the strains examined contain pentosenucleic acid and the phosphorus analyses indicated that all possess the same amount of pentosenucleic acid. On the other hand, striking differences in protein composition were found in some cases despite the fact that analyses were made for only a few amino acids. This suggested that the formation of a virus mutant involved fundamental changes in composition of the virus protein rather than simpler postulated changes (Bawden, 1943), such as a gain or loss of certain reactive groups or a rearrangement of the existing units to form a new pattern.

The amino acid analyses have now been extended by means of chemical and microbiological methods of assay so that a very much more complete picture of the composition of these strains is available (Knight, 1942, 1943, 1947b). In addition to confirming all of the previously known differences in composition among 8 different strains of tobacco mosaic virus, the microbiological assays revealed 25 new ones. Together, the analyses now account for all or nearly all of the protein of 6 of the 8 strains.

The proteins of most of the strains appear to be composed of 16 different amino acids (Knight, 1947 b). In general, it was found that those strains which, as judged from biological properties, were most distantly related to ordinary tobacco mosaic virus (TMV) differed most from it in protein composition. Also, since the differences found involved almost all of the amino acids for which analyses were made, it was concluded that mutation of TMV or of its strains can be accompanied by a change in relative proportion of any one of the amino acids of the virus protein.

No chemical distinction was found between one of these strains, the masked strain (M), and TMV. It was suggested that the difference between these strains involves differences in quantities of amino acids too small to be detected by the means employed, or that an amino acid for which analyses were not made was concerned, or that an obscure difference in the nucleic acids exists.

In striking contrast to the situation just mentioned, another strain, the Holmes ribgrass strain (HR) was found to differ from TMV in 13 respects, including the possession of 2 amino acids, histidine and methionine, which are entirely absent from TMV or the other strains. The possession by TMV and HR of the same rod-shaped structure and of the mutual characteristics upon which their relationship as strains is based seems remarkable in view of the profound difference in their compositions. This situation seems explainable only on the assumption that both strains possess a similar directive influence which exerts a compulsion in favor of a certain configuration. It was suggested that this might be one of the properties of the nucleic acid of the viruses, although there is as yet no direct experimental evidence to support the hypothesis.

The analyses of the J14D1 strain were particularly interesting because of their potential bearing on the question of how much change in chemical composition accompanies a single mutation. Since this strain appears to have arisen from TMV by 2 mutations and since only 2 differences between its composition and that of TMV were found (Knight, 1947b), it can be postulated that each mutation involved an analytically significant change in proportion of one amino acid. It was not possible to strengthen this viewpoint by analysis of the first mutant, for that had been lost, and it will be necessary to isolate a new series of successive mutants in order to make such a test.

The analyses of TMV and J14D1 also provide a basis for speculation concerning the relation of protein composition to biological activity. In Turkish tobacco, TMV produces a typical mottling, a slight distortion of the leaves and stunts the growth of the plant. Even very young plants, however, are not killed by the virus but grow to maturity, flower, and produce seed. On the other hand, the J14D1 strain produces a different set of symptoms and infection of young Turkish tobacco plants with this virus leads almost certainly to the death of every plant. The only 2 respects in which the TMV and J14D1 strains were found to differ in chemical composition were in their contents of glutamic acid and of lysine. TMV was found to contain 11.3% of glutamic acid and 1.47% of lysine, while J14D1 was found to possess 10.4% of glutamic acid and 1.95% of lysine (Knight, 1947b). It would appear from these results that there need be very little chemical difference between a virus which kills a given host and one which does not.

Amino acid analyses like those described for the plant viruses have also been made on highly purified preparations of an animal virus, influenza virus (Knight, 1947c). Microbiological assays for amino acids were made on hydrolysates of 4 to 5 preparations each of the PR8 strain of influenza A virus and

the Lee strain of influenza B virus. The results of the assays indicated that these strains of influenza virus contain approximately the same amounts of alanine, aspartic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine. However, significant differences were found in the values for arginine, glutamic acid, lysine, tryptophane, and tyrosine. It was proposed that these differences provide, at least in part, a chemical explanation for the well established lack of immunological relationship between PR8 and Lee influenza viruses. The results also suggest that the fundamental changes in protein composition which were found to accompany mutation of a plant virus may also accompany mutation of an animal virus.

An idea which has intrigued the virologist and the geneticist alike is the possibility of producing directed mutations of viruses or of genes in the laboratory. Although it is recognized that the chemistry of viruses is just beginning to develop, nevertheless the deliberate production of virus mutants by known chemical reactions or by specific radiations would seem, on the basis of the results described above, an extremely difficult task. For example, it is hard to visualize the removal of 1,300 lysine residues from, or the incorporation of 2,500 glutamic acid residues in, the peptide fabric of a fully formed virus particle by the direct action of chemicals or radiations on the virus particles themselves. Indeed, attempts thus far to produce unequivocal heritable changes by chemical or physical means have failed (Anson and Stanley, 1941; Miller and Stanley, 1941; *ibid* 1942; Kausche and Stubbe, 1939) and the main result of continued treatment with these agents has been a lethal one, that is the virus has been inactivated. However, the failure to produce heritable chemical changes in tobacco mosaic virus, for example, could possibly be attributed to a choice, thus far, of improper reagents or substituents. It is apparent that a wide variety of reagents can and should be tested. Moreover, in connection with the indecisive results achieved with radiations it should be pointed out that mutations conceivably have been obtained by these means but simply have not been detected. The random nature of radiation effects would be expected to yield only a few mutants, and with the available methods for quantitative detection of these in the case of tobacco mosaic virus, it would be very difficult, if not impossible, to distinguish between induced mutants and the spontaneously arising mutants which are invariably present in small numbers. It should be further recognized that, on the basis of present knowledge, it cannot be claimed with certainty that the virus mutations studied are, from a chemical viewpoint, typical of mutations in general. Moreover, it is not known as yet whether the chemical differences observed between virus strains are the primary results of mutation or whether they are the secondary results of critical changes which have taken place first in, for example, the nucleic acid components. If the latter is true, there would seem to be considerable justification for the assumption that some mutations can be produced by the direct action of agents on viruses (or genes) themselves. It is evident that more chemical studies on viruses are needed to advance our understanding of virus reproduction and of anomalous virus reproduction, i.e. mutation. In the meantime, any comprehensive theory of these phenomena will have to account for the striking differences in protein composition which have been observed among virus strains.

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DISCUSSION

WEISSMAN: Some time ago, Ross reported the absence of glycine in tobacco mosaic virus. He used a chemical isolation method. You have shown us a glycine content of about 2% by microbiological assay. Have you confirmed this by any other method?

KNIGHT: The presence of glycine in tobacco mosaic virus has been confirmed by application of the paper chromatography of Consden and coworkers (Biochem. J. *38:* 224, 1944). See Knight, 1947b.

WEISSMAN: It is my impression that some of the organisms employed in microbiological assay methods for amino acids do not distinguish between the l- and d-forms whereas others utilize only one of these forms. Might not the difference in activity of the several strains of tobacco mosaic virus be due to a difference in the amount of d- or l-forms of a given amino acid in each strain rather than to a difference in quantity?

KNIGHT: So far as is known, the organisms employed in our assays utilize only the natural, that is the l-amino acids and hence would not enable us to detect the presence of d-isomers. The evidence we have at present does not exclude the possible presence of small amounts of the d-forms of some of the amino acids, and this possibility should be further investigated, particularly with respect to strains like CV4 where a summation of amino acid residues and nucleic acid accounts for only about 91% of the virus. On the other hand, a number of differences among the strains have been confirmed by chemical methods of analysis which do not distinguish between d- and l-forms. Moreover, these differences are reflected by differences in physical properties such as isoelectric points. These facts combined with the summations of analyses which account in 6 instances for virtually all of the virus, lead to the conclusion that the observed differences in composition are real and that the amino acids of at least 6 strains are probably all the natural or l-variety.

PETERMANN and SCHULTZ: The constancy of the amount of proline present in the various virus mutants is of interest in the light of Pauling's hypothesis regarding the role of proline in deter-

mining protein structure. He suggested that the bond angles of the proline molecule are such as to cause a reversal in direction in the amino acid chain at the site of each proline residue. The unchanged proline content might therefore be related to the fact that the virus mutants retain their size and shape and a certain amount of immunological specificity in spite of changes in the relative amounts of the other amino acids.

HOTCHKISS: Dr. Knight has described an interesting case where two strains of a virus differ in that one has a higher content of glutamic acid and a lower content of lysine than the other, the other amino acids remaining about the same. This can be quite correctly referred to as *two* differences in analytical composition. However it is virtually impossible to surmise, or even to define, how many differences this may represent at the level of molecular structure. Quite apart from possibilities of isomerism, the removal of several hundred residues of one amino acid may be looked upon as one change, or anything up to several hundred changes in the molecule. When, now, several hundred residues of a second amino acid are added to the remainder, the number of changes may be still greater; or if perchance the second amino acid is, or may be conceived to be, replacing the first, then the net number of changes in the molecule may be construed as only one.

KNIGHT: I agree with Dr. Hotchkiss that the case he has mentioned may actually involve one or several hundred changes in molecular structure, and, I might add, could conceivably involve several thousand changes in electronic arrangement. However, the main and necessarily the primary purpose of the present investigation was not to deal with the problem which Dr. Hotchkiss has posed, but rather to determine whether there were *any* tangible chemical differences among strains of a virus. As was reported, such differences were detected in the form of striking deviations in chemical compositions, and these have been described in terms of the actual number of differences observed. It is to be hoped, of course, that the present findings may be extended to the molecular structural level when the tools become available.

FIBER STRUCTURE IN CHROMOSOMES

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It is not irrelevant, in the phase of the Symposium that is concerned with the cytological aspects of nucleic acids and nucleoproteins, to consider how they become "cytological." The problem is how they are organized into the stable microscopic structures of the cell with which they are so commonly associated. The present discussion deals with the question of chromosome structure from this standpoint. The chemical composition of chromosomes is becoming reasonably well known, and the newer advances such as have been presented to this Symposium bring few surprises. One can hope that this line of attack is leading us to a comprehension of the chemistry of genes. But the problems of the chromosome as a stable morphological entity, its mechanical properties, its behavior in mitosis and meiosis, and such questions as breakage and position effects, are much more than problems of identification of molecular species. They involve a comprehension of the intermolecular relations that create the chromosome as a *body* from the ribose and desoxyribose nucleic acids, histones and non-histone proteins. The present investigations represent an exploration of some possibilities of chromosome structure and their implications. We shall deal with (1) evidence for the existence of the proteins of the chromosomes in the fibrous state, possibly as folded molecular sheets, (2) data on the ultrastructure of protein and nucleoprotein fibers, and (3) data on enzyme reactions in the solid phase of a fibrous protein system.

1. Evidence on the Fibrous Structure of Chromosomes

The chromosome is an elongated, elastic, "insoluble" body, and the simplest way of thinking of it physically is as a fibrous protein structure, a rope of strands varying in number and configuration. It is important to note that fibrous proteins no longer are considered to be a special class of proteins. Since most "corpuscular" proteins can be converted into fibers, and at least one naturally fibrous protein, fibroin (Coleman and Howitt, 1947), has been converted into its corpuscular form, one speaks rather of the fibrous state, characterized by asymmetry and orientation along an axis and by intermolecular linkages which render the structure insoluble. We have very few chemical comparisons of the same protein in the fibrous and the corpuscular state. Goddard and Michaelis (1934) have shown that keratin fibers, which are not digestible by trypsin, can be transformed into a soluble form by reduction with thioglycolic acid. The soluble protein is digestible by trypsin. However, when the S is reoxidized, the material remains both soluble and digestible. The digestibility therefore seems to be correlated with the physical state of the protein and not with the state of oxidation of the sulfur.

In the 1941 Symposium on Quantitative Biology the senior author described an attack on the problem of the relation between chromosome composition and chromosome structure by means of enzymes. These experiments are now on a somewhat firmer basis, both because better enzyme preparations have been used and because their meaning becomes a little clearer in the light of the work on chromosome analysis reported in this symposium by Mirsky. All of the recent work has been done on salivary gland chromosomes of *Drosophila melanogaster*. Unless otherwise stated, the whole freshly dissected glands were immersed in the enzyme solution, and the smear preparations made after the digestion period.

As reported previously (Mazia, 1941), comparison of the effects of trypsin and of pepsin brings out a picture of a dual protein constitution of the chromosomes. Crystalline trypsin produces, as had been found for crude trypsin by Caspersson, a complete disintegration of the salivary gland chromosome. This is not unexpected, since trypsin is specific for peptide linkages involving basic amino acids, which would be particularly abundant in the nucleohistone type of protein and would be present in more acid proteins. *In vitro,* trypsin digests an appreciable number of peptide linkages in most proteins. Crystalline pepsin, in accordance with our earlier experiments with crude pepsin which were confirmed by Frolova (1944), produces an overall shrinkage of the salivary gland chromosomes to perhaps a third of their original volume. The results of experiments in which pepsin (5 mg crystalline pepsin per ml HCl of pH 1.5, 25° C, 15 minutes) was applied to the whole glands are shown in Figure 2 of Plate I. Figure 1 represents the control experiment, in which the pepsin was omitted. It is to be noted that the digestion reduces the volume of the interband spaces particularly, the

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chromosome contracting so that the bands are almost apposed. The bands are reduced in width. The staining is very intense in the shrunken chromosome, suggesting that the material removed by the pepsin is something other than the staining desoxyribonucleoprotein. The specificity data on pepsin (Bergmann and Fruton, 1941) inform us that pepsin would not be expected to act on peptide linkages in the vicinity of basic amino acid residues, but is specific for linkages involving dicarboxylic amino acids, particularly glutamyl tyrosine. Our own *in vitro* data on both soluble and fibrous protein substrates have confirmed, as will be seen, the expectation that trypsin will digest all the classes of proteins likely to be found in chromosomes, while pepsin fails to digest histone or nucleohistone and digests more acidic proteins.

Therefore we emerge with a picture of the salivary gland chromosome as consisting of a nucleohistone-like class of protein and another type of protein which is present in even greater volume. The latter does not participate visibly in the nucleic-acid staining of the chromosomes, accounts for most of the interband material, and also is present in fairly large volume in the bands. The digested material is probably homologous with the "residual protein" described here by Mirsky and it is interesting to note that one would predict, from his data, the presence of a large volume of residual protein in chromosomes from a highly active cell such as a salivary gland cell.

We are interested, however, in structure rather than composition. It would be concluded from Figure 2, Plate I, that the nucleohistone component comprises a continuous "skeleton" that is responsible for the continuity and banded appearance of the chromosome. That the nucleohistone portion is indeed a continuous strucure is much more clearly brought out in Figures 3 and 4 of Plate I. Here the chromosomes were first highly stretched before application of the enzyme. The technique consists of spreading in 45% acetic acid on a slide that has been coated with a concentrated agar solution and dried. When the acetic acid containing the gland is placed on the agar, the latter swells somewhat. When the preparation is pressed out, the chromosomes evidently become embedded in the agar and are stretched with it. In such preparations, the coverslip is easily removed by immersion in water; alcohol is not used. Figure 3 represents a control and Figure 4 pepsin treated material, the digestion procedure being as described above. The chromosomes being fixed, the volume changes caused by pepsin are not quite so evident as in Figures 1 and 2, but it is clear that there is no break in the structure, even where the chromosomes are highly stretched into fine strands. It is interesting to note even in the controls (and the figures only suggest the degree of stretching that can be realized by this method) that the staining desoxyribonucleic acid runs between the bands in the form of fibrous or membranous material which apparently pulls together as the interband region is pulled out, and therefore stains more conspicuously than normally. It seems valid to think of a continuous nucleohistone-like structure running through the whole length of the chromosome and expanded in the bands.

In the previous discussion of these results it was proposed that this nucleohistone continuum was the structural skeleton of the chromosome, the non-nucleohistone or residual protein providing a "matrix." In view of the observation of Mirsky that his "residual protein" retains the form of the chromosome after nucleohistone portion has been dissolved away, the possibility must now be recognized that this material too may constitute a structural continuum. Attempts to test this experimentally on the salivary gland chromosomes by extraction with concentrated salt solutions have not been effective. The difficulty lies in the fact that salivary gland chromosome structure is very sensitive to pH changes in the neutral and alkaline range; above a pH of 4 to 5, the chromosomes become dispersed into a mass of fine threads (Mazia, 1941). In our experiments we have tested the action of 1 M NaCl on Drosophila salivary gland chromosomes over a wide range of pH, and have found no effects that were not observable after the action of dilute buffers at the same pH. Since the salt extraction is not very informative, and we do not have an enzyme available that will digest histones and will not digest more acid proteins, we cannot, employing the salivary gland chromosomes which would be ideal from the cytological point of view, study directly the structure of the residual protein component in the absence of the nucleohistone. However, from the pepsin experiments it is clear that the residual protein runs through the whole chromosome. Both bands and interband regions are reduced in volume after pepsin digestion. It no longer seems unreasonable to think in terms of a truly dual structure, of a nucleoprotein continuum running through a non-nucleoprotein continuum in such a fashion that either component can maintain the form of the chromosome, both contributing to its mechanical properties, and the proportions of the two determining to some extent the volume of the chromosome.

We reported previously that cathepsin solutions in the form of tissue autolysates did not seem to affect chromosomes, and suggested an interpretation in terms of the physical organization of chromosome proteins. We can now report observations on the action of concentrated cathepsin prepared from beef liver by the method of Anson (1937). This material has been tested for proteolytic activity on hemoglobin substrate by a standard method (Anson, 1937) and on a chromosin substrate by measuring the amount of nucleic acid rendered soluble in water.

Liver cathepsin prepared by Anson's method was preserved in frozen-dried form. The solution used in these experiments

contained 10 mg of dried material per ml, whose protein content was represented by 0.32 mg "total tyrosine color value" (Anson, 1937). The material, which contained considerable ammonium sulphate, was dissolved in 0.5 M NaCl and adjusted to pH 3.5 with acetic acid. NaCN or cysteine hydrochloride was added to a final concentration of 1 mg per ml. The determinations on hemoglobin substrate followed Anson's method exactly. Our solution had an activity of 52×10^{-4} Hb units per ml at 35° C. For estimation of activity with nucleoprotein substrate, a solution of chromosin from calf thymus containing 10 mg of desoxyribonucleic acid per 5 ml was used. The substrate was adjusted to pH 3.5 with acetic acid, forming a rather soft gel at this pH. One ml of enzyme solution was added and the mixture was incubated at 35° C. The control consisted of 5 ml of the substrate which was incubated at the same time, and to which the enzyme was added at the end. After 24 hours, 6 volumes of water were added, the mixtures adjusted to pH 6, and centrifuged. The precipitates, which were presumed to be the undigested chromosin, were redissolved in 1 M NaCl and aliquots analyzed by the diphenylamine reaction (Seibert, 1940). In the control, 9 mg of nucleic acid were recovered, while in the enzyme-treated mixture only 1 mg was recovered. Therefore the enzyme, which was quite active against hemoglobin substrate, was also active enough to digest most of the nucleoprotein substrate in 24 hours.

The results of the action of this enzyme preparation on fresh salivary glands of *D. melanogaster* is shown in Figures 5 and 6, Plate I. Figure 6 is the control, treated with all the constituents of the enzyme mixture except the cathepsin for 30 hours. The poor quality of the preparation by cytological criteria is expected after such treatment. Exposure to the enzyme (35° C for 30 hours) not only seems to have no microscopically observable effect on the chromosomes, but, because of the digestion of extrachromosomal material, results in a much better preparation, cytologically. Variations of time, enzyme concentration, and presence or absence of activator all failed to produce evidence of digestion. It seems unlikely, from these observations, that in experiments with impure enzymes, such as tissue "nucleases," the cathepsin contamination is as serious a source of error as Catcheside and Holmes (1947) have suggested.

In interpreting these results, the possibility appears that the digestibility of the chromosomal proteins is determined by their physical organization as well as by their composition with respect to peptide linkages. The alternative is that these proteins are grossly different in composition from the substrates tested *in vitro*, which is not appealing in view of the volume of information on chromosome composition that has been presented by Mirsky and Pollister, and the fact that Bergmann and Fruton found that the cathepsin prepared by Anson's method from beef spleen had, in the presence of activators, a very broad specificity covering most varieties of peptide linkages.

The most common hypothesis of chromosome structure is that the proteins are in a fibrous condition. We can exclude the possibility that insolubility alone prevents catheptic digestion in view of the work of Maver (1939) on the digestion of precipitated liver globulin. If neither peptide composition nor insolubility determine the resistance of the chromosomes to cathepsin action, the explanation may be sought in the structural configuration responsible for fibrous properties. We therefore undertook a study of the action on protein fibers of the enzymes that had been employed in the chromosome experiments. Of the methods of converting globular proteins to the fibrous condition we chose the Devaux (1935) technique of spreading the globular protein as a monolayer and compressing the monolayer into an insoluble, elastic fiber. Details will be presented in a later paragraph. This method, in contrast to those involving heat, urea, or detergents, seemed to be within the bounds of physiological possibility. For the special case of nucleoproteins, we also used the Mirsky and Pollister method of precipitating chromosin fibers in dilute salt solutions. The enzyme was applied to the fibers and visible digestion was used as a criterion of enzyme action.

Crystalline trypsin and crystalline pepsin digested the artificial fibers exactly as predicted from their behavior in solution. Surface-compressed fibers of hemoglobin, egg albumin, histone, and fibers of chromosin prepared by both methods digested rapidly in trypsin. The nucleoprotein fibers prepared by either method showed no effect of pepsin, nor did histone fibers prepared by the surface method. The other proteins tested were digested by pepsin. It was possible, by the surface method, to prepare fibers composed of a mixture of nucleoprotein and albumin and to observe that pepsin digested only part of the fiber, in analogy with the action on chromosomes.

The cathepsin solution described above digested none of the proteins in the fibrous form. The experiments were carried for as long as 48 hours at 35° C. In the case of chromosin, there was no difference between the behavior of fibers prepared by spreading and those prepared by precipitation. Considering the experiments on isolated proteins alone, we might say that we have here a criterion for distinguishing between the corpuscular form and at least one fibrous form. There is a precedent in the analogous work of Goddard and Michaelis. Obviously, an even wider variety of proteins would have to be tested to establish this as a general test. If it is valid, then its applicability to the chromosome proteins would be no less legitimate than any case where one draws a conclusion from a negative reaction to a chemical test. At least it can be said that the parallelism between the nondigestibility of chromosomes and that of fibers composed of the classes of proteins believed to be present in chromosomes, the activity of enzymes on similar proteins in solution having been demonstrated, offers presumptive evidence of

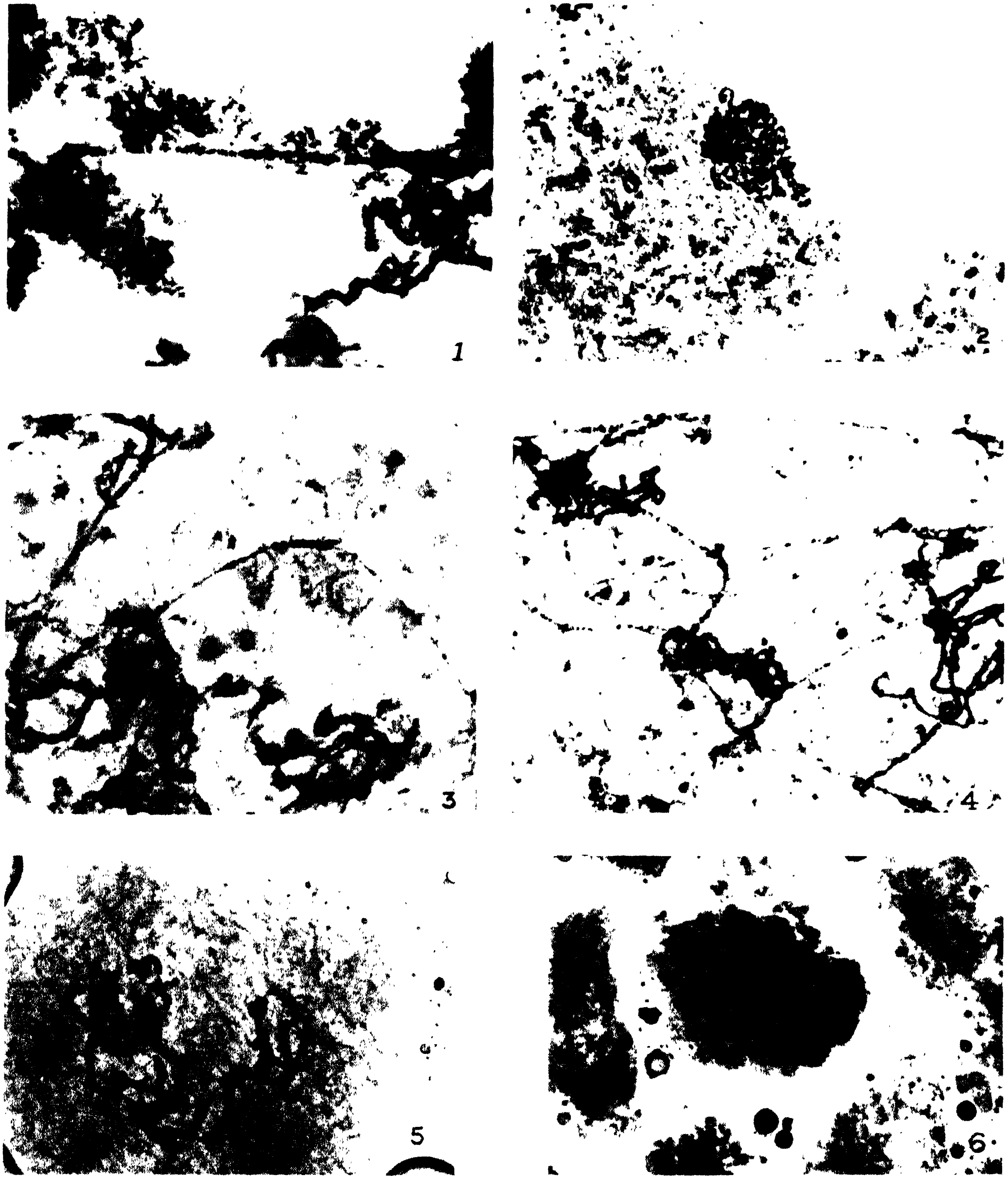


PLATE I

FIG. 1. Chromosomes from control gland; 15 minutes in HCl pH 1.5.
FIG. 2. Pepsin-treated chromosomes. Whole gland exposed to 0.5% crystalline pepsin at pH 1.5, 25°C.
FIG. 3. Chromosomes from gland fixed in acetic acid, stretched on agar, and treated as in Fig. 1.
FIG. 4. Chromosomes fixed and stretched on agar and digested with pepsin as in Fig. 2.
FIG. 5. Chromosomes from gland treated with cathepsin solution for 30 hours at 35°.
FIG. 6. Control to Fig. 5, exposed for 30 hours at 35° to cathepsin-free buffer-activator mixture. Acetocarmine preparation.

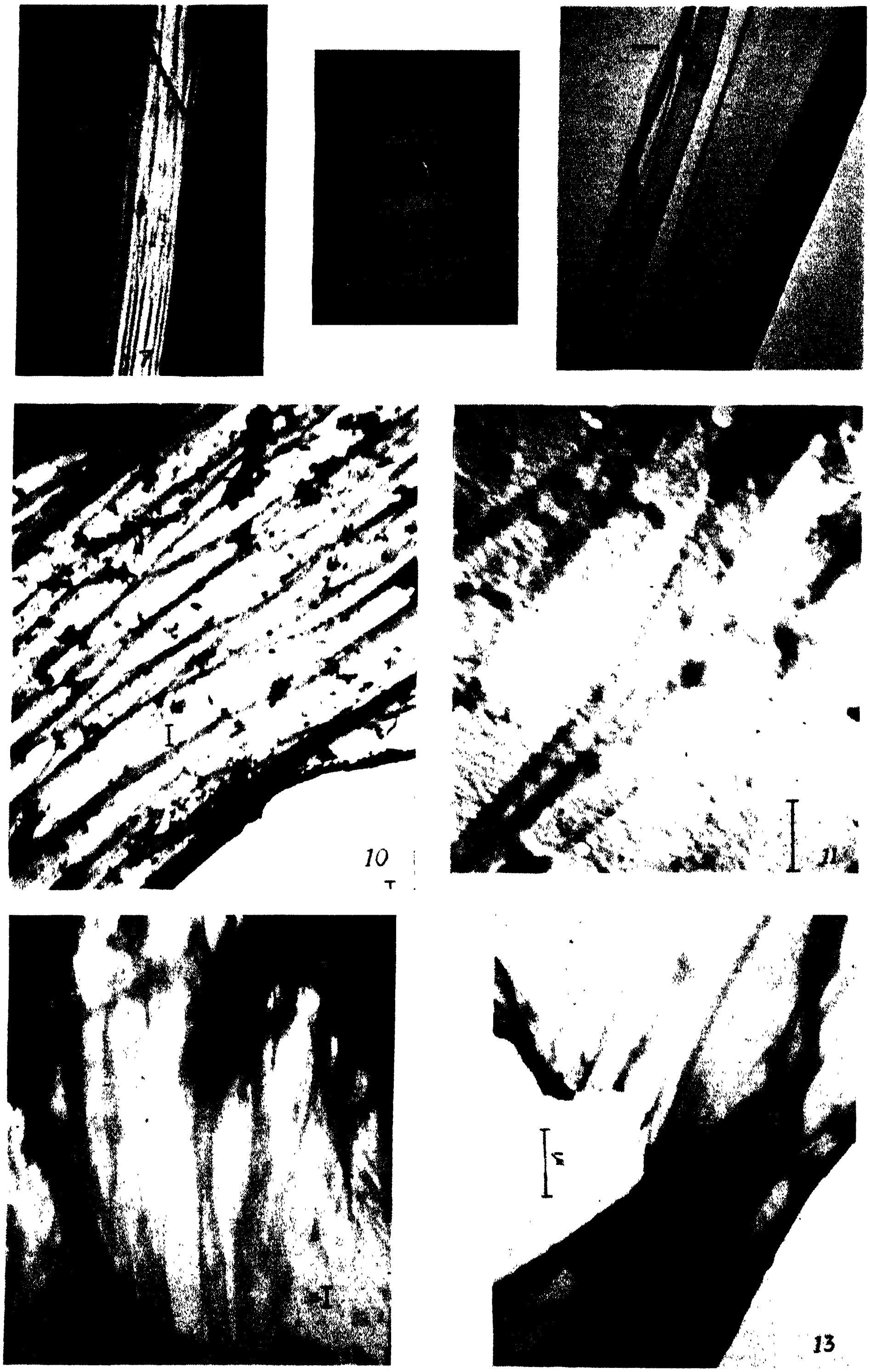


PLATE II

FIG. 7. View of surface-compressed ovalbumin fiber through crossed Nicols. × 250.
FIG. 8. X-ray diffraction of wet surface-compressed fibers of ovalbumin. Reduced 35%.
FIG. 9. Electron microscope view of edge of surface-compressed ovalbumin fiber. Uncast.
FIG. 10. Electron microscope view of surface-compressed thymus chromosin. Gold cast.
FIG. 11. Same as Fig. 10, higher magnification.
FIG. 12. Electron microscope view of region of fiber of thymus chromosin precipitated in water. Gold cast.
FIG. 13. Region of precipitated thymus chromosin fiber at higher magnification. Gold cast.

the fibrous state of the proteins in the chromosomes. It is to be noted that there is no shrinkage of salivary gland chromosomes in the cathepsin; the evidence of the fibrous condition applies to both the nucleoprotein and the pepsin-digestible protein.

The term fibrous has been defined rather loosely. These experiments do not distinguish between the fibers prepared from surface films and, in the case of nucleoprotein, the fibrous precipitates. In the next section, we shall present evidence showing that the distinction, in terms of structure, may not be very significant. It is meanwhile noteworthy that the non-nucleoprotein behaves toward cathepsin just as the nucleoprotein, which would imply that it is either a protein of the myosin type in the form of a fibrous precipitate or that its formation is analogous to that of the surface fibers.

2. Structure of Protein Fibers

If there be justification for thinking of chromosomal proteins as fibrous, this structure has certain implications for interpretation of chromosome function. Our problem is the visualization of the molecular order which makes for the mechanical properties of chromosomes. We have undertaken a study of the structure of artificial protein fibers with a view to such a visualization.

Artificial fibers of various proteins were prepared by introducing drops of 1% protein solutions to the surface of a Cenco Hydrophil Balance tray containing appropriate buffers. Usually we added to the solution about 0.5% isopropyl or isoamyl alcohol to assist spreading (Derivichian, 1939). The film thus formed was compressed between waxed chromium barriers until these were about 1 cm apart. The resulting fiber could be picked from the surface by the tip of a needle. Albumin was spread over McIlvaine buffer of pH 4.2. Chromosin fibers were prepared by spreading over buffer of pH 6.0, using solutions in 1 M NaCl and also aqueous solutions to which just sufficient alkali was added to dissolve the material. For electron microscopic observation, the clean screens, without previous collodion coating, were placed under the compressed film and lifted through the surface. The mounts were then frozen-dried. For X-ray diffraction, long fibers removed from the surface were wound, under tension, around a bow-shaped frame until a bundle about 0.5 mm thick was obtained. The bundle was stretched about 30-50% and frozen-dried.

The microscopic appearance of part of an egg albumin fiber is shown in Figure 7, Plate II. The appearance suggests fibrillar composition. This is even more strongly suggested in surface-compressed fibrous chromosin, which is not figured. Figure 7 was taken through crossed Nicols, and shows the birefringence which was observed in the fibers of all the proteins used. Measurements by Dr. Edmund E. Marshall yield a birefringence value of 0.002 for the wet fibers, positive with respect to the fiber axis. Correction for water content would probably raise the value considerably.

The electron microscope reveals that the apparent fibrillar structure of the fibers is an illusion, that they actually remain sheet-like, the "fibrils" resolving into folds of the sheet running roughly perpendicular to the direction of compression. Figure 9, Plate II shows the appearance of a portion of an albumin fiber, Figure 10 and 11 represent chromosin fibers prepared by the surface technique and gold-cast. The material again is essentially in the form of a thin sheet. A peculiarity of the nucleoprotein preparation is the appearance of anastomosing thickened regions which do not appear to be folds. The figures represent an extreme example; in other preparations the bands are few or absent. Figure 12 represents the more common appearance at high magnification. We have no interpretation to offer of the apparent heterogeneity of nucleoprotein fibers in these cases. The most obvious hypothesis would be that they are regions of concentration of conjugated nucleoprotein while the thinner material between would be protein alone. Mirsky and Pollister (1946) report that chromosin films prepared from solutions in 0.02 M NaCl are 15-16 A thick while those of histone alone have a thickness of 8-9 A. It is possible that our starting solutions of the high polymer form of chromosin contained both associated and dissociated nucleic acid and protein. In any case, the sheet-like structure of the fiber is very clear. We cannot answer the question whether the unit thickness observed in all the folded compressed films is monomolecular. We have no method of measuring the thickness, and can only state that the observations do not preclude the possibility that we are here observing single unfolded protein layers. It is certain that the films are extremely thin and almost unobservable, except where folded, without gold-casting.

In further analysis of the structure of these fibers we have studied the wide-angle X-ray diffraction of bundles of parallel albumin fibers. Figure 8 shows representative results of a series of exposures employing radiation of 1.54 A in a camera of 5.377 cm radius. In no case have we observed any evidence of orientation. We generally obtain two rings corresponding to spacings of 4.22 A and about 3.2 A, and in one case an additional ring at 3.8 A. There is a suggestion of a larger spacing at 10-12 A, but this is difficult to verify by our method. These spacings are of the order of magnitude of those expected for a beta keratin structure, but the important point is the absence of evidence of orientation. Considering the strength and elasticity of the fibers, which we plan to measure, it is to be concluded that a fibrous organization of proteins does not require parallelization or simple folding of polypeptide chains. This, of course, allows the retention of any degree of specific folding that is permitted by the two dimensions of the film, and therefore presumably retention of "native" properties. The most reasonable conclusion is that protein fiber structure is not restricted to a polypeptide grid, but may be

based on chains or grids of whole molecules which become linked when they are opened up by the surface spreading. It is difficult to reconcile this with the statement of Astbury and Bell (1938) that built-up albumin layers show a typical beta keratin pattern, but this pattern is hardly to be discerned in their published figure. If molecular grids constitute the fibers, their pattern might be observed by means of small-angle diffraction, and such work is now in progress in collaboration with Dr. George W. Clark of the University of Illinois.

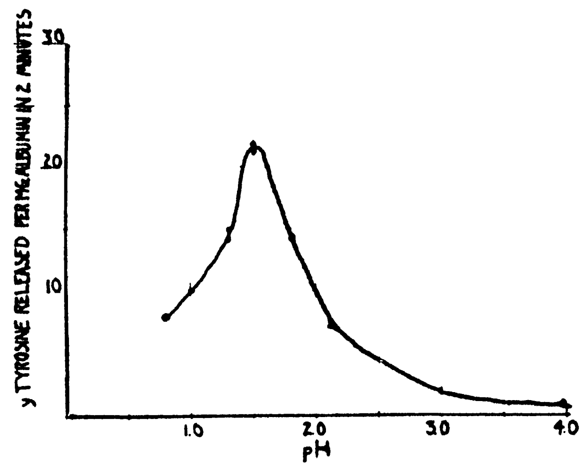
Summarizing our observations of the structure of artificial fibers prepared by surface spreading of corpuscular proteins it is seen that the apparent fibrillar structure of the fibers resolves, under the electron microscope, into that of a folded sheet, possibly of monomolecular thickness. Although these fibers are composed of oriented units, as evidenced by the polarized light observations, X-ray diffraction indicates that this orientation does not imply a polypeptide grid structure analogous to the keratin picture. The spacings characteristic of keratin show a randomness that permits postulation that a specific folding pattern may be preserved within the two-dimensionalized molecules and that the fibrous properties and birefringence may be based on a molecular grid or chain.

3. Enzyme Action in Fiber Systems

The possible parallelism between fiber protein structure and chromosome structure raises the questions of the compatibility of such structure with the specific activities, particularly enzyme activities, presumed to take place in chromosomes. If the compatibility is established, the problem of the quantitative and qualitative characteristics of reactions in such a "solid" system require investigation, since our conceptions are largely based on the study of solutions. Observations such as those of Krugelis (1946) suggest very clearly the existence of cases where both enzyme and substrate are part of the chromosome structure itself. These questions have been investigated in the simple case of fibers composed of egg albumin and pepsin, with crystalline preparations as the starting material.

Solutions of crystalline egg albumin and crystalline pepsin containing 10 mg of protein per cc of McIlvaine buffer of pH 5.0 and about 0.5% of isoamyl alcohol were spread by placing a drop on the surface of diluted McIlvaine buffer of pH 4.2 in a waxed trough. After 30 seconds (longer times made no difference) the film was compressed between barriers and the fiber removed to distilled water. Masses of fibers thus collected were washed and frozen-dried. For quantitative estimations of digestion, 1 mg samples of the material were placed in 0.3 ml of HCl of pH 1.5, at 25° C and the reaction was stopped at intervals by addition of 1 ml of 10% trichloroacetic acid. The material was filtered and the whole filtrate was analyzed for acid-soluble tyrosine plus tryptophane plus cysteine by the method of Anson (Northrup, 1938), which was not varied except for proportional reduction of volumes of reagents. Color was read photoelectrically, and compared with a tyrosine standard. Our data are therefore given as "mg tyrosine color equivalents" (Anson, 1937). The numerous details of fiber preparation, equilibration, stirring, filtration, and analysis will be given in another publication.

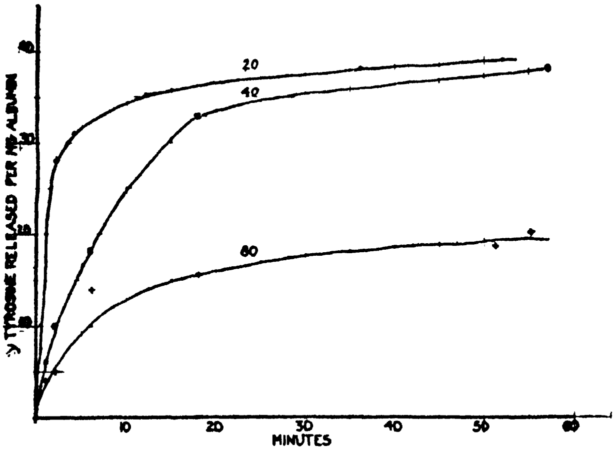
The first question that arises is that of the possibility of reaction between enzyme and substrate when both are incorporated into the fiber. This is easily tested microscopically by placing the fibers in acid solution (HCl pH 1.5). They are observed to begin to disintegrate immediately and finally to disappear. It is evident that we are actually observing digestion of substrate by enzyme in the solid phase. Although final proof depends on the chemical determinations of digestion products, it should be pointed out that this microscopic measurement of the time required for complete digestion has always checked with the chemical measurements and is in some ways more reliable as an indication of that part of the reaction that takes place in the solid phase.



Text Fig. 1. The pH-dependence of pepsin activity on ovalbumin in a fibrous complex system. Ordinates—Total digestion products measured as "milligrams of tyrosine color equivalents" per mg ovalbumin after two minutes of digestion. (A "milligram tyrosine color equivalent" is that amount of split products which gives the same color value with the Folin-Ciocalteau reagent as one mg of tyrosine.)

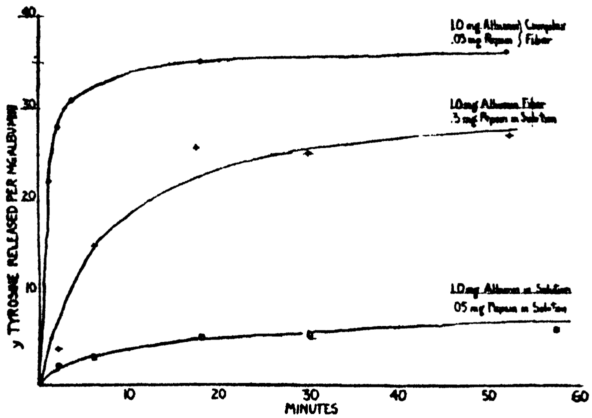
The quantitative determination of split products containing tyrosine, tryptophane or cysteine leaves no doubt that we are dealing with proteolysis. Such data are shown in Text Figures 1 and 2, which will be discussed. In general, the time of disappearance of the fibers corresponds to the time of liberation of 50 to 80% of the total split products. Text Figure 1 shows that the pH-activity relationship is one characteristic of the digestion of albumin by pepsin when both are in solution. The same pH dependence is found when the microscopic criterion is used. Thus there is no gross evidence that the physical state of the system has affected its hydrogen ion

dependence. Text Figure 2 shows the time course of digestion and the relation between the rate of digestion and the ratio of substrate to enzyme in the fiber. The ratios are based on weights of material in the solution. Film-area measurements which will be presented elsewhere justify this assumption, at least approximately. It is evident that the rate of the reaction depends on the proportion of enzyme and substrate molecules. When the experiments are run for longer periods of time, the fibers containing albumin to pepsin ratios of 20-1, 40-1, and 80-1 digest to about the same extent. Considering the sizes of the pepsin and albumin molecules, it is somewhat difficult to imagine how a pepsin molecule can be in contact with 20 or 40 albumin molecules, yet the data indicate that at least 50% of the ultimate total split products are released before the fiber disintegrates. This simple explanation, that once a pepsin molecule liberates itself it acts in solution, is not valid in its simplest form because, as Text Figure 3 will show, this amount of pepsin in solution could not account for the rate of the reaction. A refinement of this explanation is that the liberated pepsin is trapped in the fiber mass and operates in a small volume and high effective concentration. If this were so, the smallest fibers should digest more slowly than larger ones, since there would be more opportunity for the enzyme to be dispersed into the external medium. This is not the case. However, the decisive experiment that is planned is to follow by means of the electron microscope the reaction of very thin films in a large volume of medium. In such a case, the liberated enzyme molecules should be removed, leaving holes, and the albumin film will remain. We have performed in a preliminary way the analogous experiment of following by the surface balance the area of a pepsin-albumin film spread over an acid solution. The evidence indicates complete digestion.



TEXT FIG. 2. Time-digestion curves of ovalbumin-pepsin fibers at various substrate-enzyme (S/E) ratios. Ordinates—Total digestion products measured as "milligrams of tyrosine color equivalents" per mg ovalbumin.

If these explanations prove incorrect, the data on digestion at high substrate-enzyme ratios would require explanation along the lines suggested by Rothen (1946, 1947), who proposes that an enzyme molecule has a considerable range of action, and need not be in contact with its substrate. The genetic significance of such a theory is rather obvious to those concerned with interaction of genetic loci and we propose to investigate this question in our system.



TEXT FIG. 3. Time-digestion curves comparing pepsin activity on ovalbumin under various substrate-enzyme relationships. Ordinates—Total digestion products measured as "milligrams of tyrosine color equivalents" per mg ovalbumin.

Investigating the particular properties of the solid-phase enzyme system, we have compared it with systems containing the same amounts of pepsin and albumin in the same volume of medium, but where the pepsin in solution acts on fibrous albumin or where both are in solution. Such data are presented in Text Figure 3. It is evident from the data that digestion proceeds much faster when enzyme and substrate are both contained in the solid phase, the total amounts and volume being kept constant. The obvious explanation is that we are dealing with a high enzyme "concentration" in the fibers, but the term concentration in its chemical sense does not seem applicable to this system. In any event, it can hardly be said that "surface denaturation" diminishes the specific activity of the enzyme.

In some of the earlier discussions of surface denaturation of enzymes (Langmuir and Schaeffer, 1938) incomplete spreading of some of the molecules was considered the major source of error, partly because the measured activities were low. The more recent data (Langmuir and Waugh, 1938; Seastone, 1938) seem to establish the fact that proteins either spread completely or go into solution. In our own

case the activity in the film was much higher than predicted. Area measurements to be presented elsewhere indicate that at pH 4.2 and with our technique the albumin is spread to an extent of about 0.85 square meters per mg, and the figure for pepsin is about 0.7. While this is short of the best figure obtainable, it still would not permit the presence of very much unspread pepsin in the film. We have checked the adsorption of pepsin from the trough onto the films by injecting pepsin solution under albumin films. The compressed fibers showed no activity at pH 1.5, indicating that the film does not adsorb pepsin in a form in which it cannot be removed by the repeated washings we have employed.

4. Discussion

This investigation was designed to test the implications of one conceivable type of structural organization relevant to chromosomes. The comparative digestion studies on chromosomes and on proteins in the corpuscular and fibrous state can only be claimed to demonstrate the compatibility of chromosome structure with the structure shown by the fibers. Even if the evidence were stronger, we should be anxious to avoid applying the discredited term "model" to the relation between fibers and chromosomes, but rather consider the fibers as material for studying the implications of fibrous protein structure. At worst, they represent a system for studying the properties of enzyme reactions in a situation where molecular mobility is limited by "solid" structure, and where kinetic concepts such as concentration and collision-probability have different implications from those derived from solution studies. The data indeed show that the rate of the pepsin-albumin reaction cannot be predicted from the amounts of the reactants without specifying their physical relations.

As biologists we are interested in visualization of the structural situation in which phenomena such as reduplication, synapsis, crossing over, intergenic reactions, breakage and recombination occur. The implications of our data for this problem of visualization are apparent. First, the cell could conceivably manufacture fibrous structures at interfaces even from low molecular weight proteins. In this connection, suggestions of persistent association of the chromosome complex with the nuclear membrane are of interest (Vanderlyn, 1946).

The structure data indicate that it is not necessary to adopt the Astbury picture in order to obtain a mechanically sound fiber, and the enzyme activity data indicate that there is no incompatibility between the fibrous state and the native state in its functional sense.

Second, if chromosomal proteins are patterns or templates for their own reduplication and for the production of exactly reproduced products, simple thinking requires that they be essentially two dimensional at the molecular level. Our data indicate the compatibility of fibrous appearance at the microscopic level with an essentially two-dimensional structure as observed at the molecular level. That the unit sheet in the fibers actually has the thickness of an unfolded protein molecule remains only a definite possibility until we find a means of quantitating our electron microscope observations. Third, the possibilities of long range interaction of molecules in the film continuum, suggestive of the ideas of Szent-Gyorgyi (1941) and consistent with the observations of Rothen, might bear on the interactions evidenced in the data of genetics.

Finally, we may refer to our current investigations of the inactivation of pepsin by X-rays in pepsin-albumin films. The effective doses are well within the physiological range and higher by orders of magnitude than the doses required to affect pepsin in the pepsin-albumin solutions used as a starting material for the films.

Finally, since the nucleoprotein soluble in strong salt and precipitable as fibers has been discussed in this symposium, we may consider the relation between the surface fibers and the fibrous precipitates. We have been particularly interested in the surface fiber structure because it is applicable to many proteins independent of their size, form, or nucleic acid content when in solution and because of the implications of the two-dimensional structure. Very recently we have compared these with the fibrous precipitates under the electron microscope. Figures 12 and 13 show low and high power views of fibrous precipitates. They were prepared merely by stirring a small drop of chromosin solution into water and picking up the precipitate with an electron microscope object screen. The figures show only some interesting samples of the observations. The observations have revealed that the fibrous precipitate is also in the form of a folded, very thin sheet. At points of minimum folding, such as shown in Figure 13, the sheet appears as thin as that of albumin or other surface-spread proteins. We can see no reason at present for distinguishing between the general class of fibers formed by interfacial unfolding of protein molecules and the special type represented by the high molecular weight desoxyribonucleoprotein. The minimum residue of our observations is that the fibrous properties of chromosomes are consistent with the essentially two-dimensional structure required by the simplest picture of the reduplication mechanism, and that such a structure is compatible with specific protein activity.

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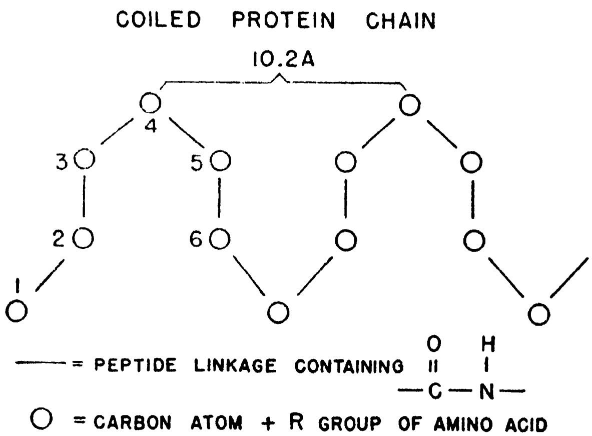
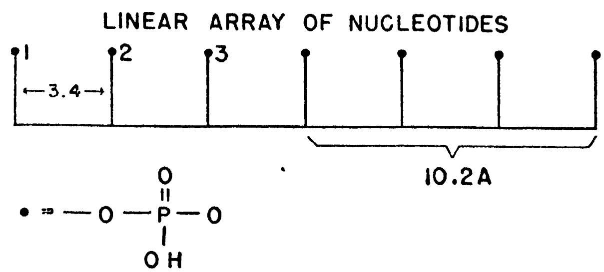
DISCUSSION

COHEN: I wish to suggest a mechanism for the inability of cathepsin to disrupt chromosome structure, as found by Dr. Mazia, assuming that his cathepsin actually split off amino acids from the chromosomes, The hypothesis arises from considerations of the structure of thymus nucleohistone. This material, isolated by distilled water extraction of thymus and precipitation by 0.14 M NaCl, has a protein and nucleic acid content such that there are about 2 amino acids per nucleotide. In nucleoprotamines, the stoichiometric relations indicate 1 amino acid per nucleotide. In the latter instance, it has been observed by Astbury that the internucleotide spacings of 3.4 A approximate closely the interamino acid spacings. A simple linear alignment of basic amino acid groups and acidic nucleotides (1:1) provides the weight relations found in the nucleoprotamines.

The histones are heat denaturable and hence not fully extended molecules in their native state. This mode of existence may be simply represented by the Huggins picture of a coiled condensed α-keratin. The spacings of this model, as determined by X-ray, are illustrated with its possible sites of interaction with nucleic acid as follows:





Thus for a spacing of 10.2 A we have 6 amino acids and 3 nucleotides or the approximate stoichiometric relations which one finds in thymus nucleohistone. If this is actually the mode of organization and interaction within this material one should be able to obtain X-ray data in confirmation of this picture, since the 10.2 A spacing or its multiple should be far more marked than in simple proteins. It appears possible that the technique of Dr. Petermann in inhibiting desoxyribonuclease during the preparation of the material may permit the isolation of fibrous "native" nucleohistones which are more suitable for detailed X-ray analysis.

Dr. Ris pointed out that treatment of chromosomes with desoxyribonuclease liberates histone as well as nucleotides. That the smaller histone mole-

cules are organized by the larger nucleic acid molecule is suggested by other data as well. This model provides a mechanism whereby a large proportion of the protein in groups of 1-5 amino acids out of a possible 6 may be split off without appearing to alter the basic structure of the molecule. Furthermore, a protein hole of a specific size bounded by specific amino acids presents a useful framework for the regeneration of the original contents. Thus the model suggests a mechanism by which proteolytic and proteogenic enzymes may be removing or replacing specific groups of amino acids possibly capable of influencing biochemical events in the cell without altering the basic framework of the largest portion of the chromosome, the nucleohistone.

Far from disrupting the basic structure of the system, there is even evidence that proteolysis results in the reinforcement of the structure. We found that proteolysis of the relatively nonviscous thymus nucleohistone resulted in great increases in viscosity. As protein pieces were split off, the newly uncovered nucleotides regained the ability for intermolecular interaction and the structural properties of the solution were increased. It seems to me that these phenomena observed in experiment and appearing reasonable from theoretical considerations may provide an explanation for Dr. Mazia's observation.

PETERMANN: Is it not possible that the very rapid release of "tyrosine" in a form soluble in trichloracetic acid from albumin pepsin fibers is due to the fact that the secondary bonds of albumin have already been broken during the formation of the film? The splitting of peptide bonds in unfolded polypeptide chains might give rise to trichloracetic acid soluble fragments much more rapidly than would the splitting of the same number of peptide bonds in the highly cross-linked albumin molecule.

MAZIA: Our Text figure 3 would confirm Dr. Petermann's suggestion as far as the relative digestibility of fibrous and dissolved albumin by dissolved pepsin is concerned, and we had just that in mind when we set up the experiment the way we did. The object of the experiment, however, was to compare pepsin in the fiber with pepsin in solution when the albumin substrate was the same in both cases, namely fibrous albumin. Comparing the upper two curves, we see that the pepsin in the fiber is more active than that in solution even when the state of the albumin was not a variable. I should like to repeat that I am not sure what we mean when we say the pepsin is "more active" in one configuration than the other, except in relation to a particular experiment. The major problem, and the one most relevant to our thinking about the cell, is precisely that we have no way of comparing activities in different physical states, since we cannot apply the concept of a "concentration."

THE NATURE OF THE INTERACTION OF NUCLEIC ACIDS AND NUCLEI WITH BASIC DYESTUFFS

L. MICHAELIS

Introduction

Since Paul Ehrlich's research in the field of histological staining methods sixty years ago, it has been known that cellular nuclei can be stained with basic dyestuffs. This property of the nucleus was always recognized as due to its content of nucleic acid. In this paper it will be shown that the particular manner in which basic dyes are adsorbed by nuclei or by solutions of nucleic acids is something quite specific, and different from the manner in which such dyes are adsorbed by other stainable substrates that may occur to biologists in the widest sense. An attempt will be made also to correlate this property of nucleic acids with their structural configuration.

The progress in fundamental knowledge and methods since Ehrlich's time which allows us to attack the problem once more, is as follows. First of all, we know now that nucleic acids occur not only in the nucleus but also in the cytoplasm. We know now that the basophily of several cytoplasmic structures, such as the cytoplasm of the liver cell, or of the lymphocyte, can be attributed to nucleic acids also. In the second place, we are now in the favorable position of studying rapidly and precisely the absorption spectra of dyestuffs with our modern photoelectric spectrophotometers, such as the well-known Beckman instrument.

The cell contains nucleic acid not in the free form but attached to proteins or protein-like compounds as "nucleoprotein." Nevertheless, the staining of uncombined nucleic acids, as they occur in aqueous solution, and the staining of cells and nuclei, are dealt with here on the same footing. The justification of this procedure must be commented upon first of all.

No doubt, the adsorption of a dyestuff cation by nucleic acid takes place by means of the phosphoric acid group of nucleic acid. The opposite electric charge is a prerequisite of staining, although it is not a sufficient condition. The sodium ion also has a positive charge, but it cannot be considered as "adsorbed" by nucleic acid. Other structural properties are responsible for the adsorbability, but they will not be discussed here. Opposite electrical charge is a condition not sufficient for adsorption. If a nucleoprotein is stained with a basic dye, the dye cation must replace the positively charged ion to which the phosphoric group may have been attached previously.

To be sure, sometimes dyestuffs are adsorbed by a substrate in disregard of simple Coulomb forces. For instance, Klotz has presented spectrophotometric evidence that some anionic azo dyes can be adsorbed by certain (not all) proteins under conditions where the latter are also negatively charged. Such a possibility seems to prevail especially for such dyestuffs, which have a very great tendency to become colloidal under many conditions, especially in the presence of even low concentrations of neutral salts, *e.g.*, some azo dyes and especially tetrazo dyes such as congo red. This is, however, not the case for thiazine dyes with which we are dealing here essentially, and many others. In histological staining, a stained tissue slice releases a part of the dye by extraction with alcohol. Probably, what alcohol can extract is that part of the dye adsorbed in excess of the ion-exchange mechanism. If staining with a basic dye is due to cation exchange, only the cation of the dye is fixed by the substrate. Staining in excess of cation-exchange involves adsorption not only of the dye cation but also of an equivalent amount of some anion. On staining nucleic acid with basic dyes, practically only the cation-exchange reaction has to be taken into consideration.

There is a competition between various cations for adsorption by the negatively charged phosphoric group of the nucleic acid. This competition can be demonstrated as follows. When a slice of a fixed tissue, or a fixed smear, is stained, say, with methylene blue and the excess of the dye is washed out first with water and then with alcohol, there will very soon be reached a condition where practically no dye can be further extracted either with water or with alcohol. But the adsorbed dye can be extracted by acids—the hydrogen ion competes with the dye cation. Or, the adsorbed methylene blue can be extracted with, and replaced by any other cationic dye. The blue slice, immersed in a solution of Bismarck brown, gradually releases methylene blue and it is stained with the brown color of the other dye. Or, when the slice is first kept in a solution of quinine sulfate, washed with water, and then immersed in a solution of methylene blue, the slice is stained blue, not immediately, but very gradually, which indicates that quinine had been adsorbed and is being gradually replaced by methylene blue.

The nucleus of the cell in the living state, immersed in a very dilute solution of any basic dye, is not stained at all. This phenomenon, long known, can be best interpreted by the fact that the nucleic acid is attached to the basic groups of a protein,

and the dye, when present in the form of a highly diluted solution, cannot compete with the protein. The dye, when present in higher concentration, does stain the nucleus by replacing the protein, but this is an irreversible process. The nucleus is killed as it is stained. It seems to me that such a consideration both explains why the living nucleus is not stained at all by a dilute dye solution as long as it is alive, and why the nucleus as soon as it *is* stained, is no longer a living nucleus. On fixation, obviously some irreversible change, let us say a denaturation, has happened to the nucleoprotein such that the combination of the nucleic acid and the protein is looser and the competition of a dye is stronger. So we see why the staining of a solution of nucleic acid and of the fixed tissue slice can be understood from the same principles. In either case, the staining consists of the attachment of the dye cation to the phosphoric acid group of nucleic acid.

There was never any doubt as to the fact that the basophily of the cellular nucleus is due to the nucleic acid. However, Ehrlich observed that not only is the nucleus basophilic, but that there are other basophilic histological elements too. First of all, in some cells, the cytoplasm also is basophilic. We know today, that this basophily also may be due to nucleic acids. We know today that the nucleic acid of the nucleus is essentially desoxyribonucleic acid, and that of the cytoplasm is ribosenucleic acid. There is no essential difference in the behavior of these two kinds of nucleic acids when exposed to a plain solution of a basic dyestuff. This does not preclude that there are certain staining methods which can differentiate those two kinds of nucleic acid. We are not concerned with those here. Anyway, the solution of any one basic dyestuff shows the same behavior toward any kind of nucleic acid, independent of their special chemical nature, and also independent of the molecular size of the nucleic acid. As far as one may say today, a clear solution of a preparation of yeast nucleic acid of low molecular weight, behaves in this respect as a very high-molecular, viscous and turbid solution of thymonucleic acid. We are dealing here with a fundamental property of all nucleic acids, which does not seem to be shared by any other colloidal subtrate.

However, there are certain histologic elements which are indeed basophilic, but behave differently from nucleic acid. These can be distinguished by the shade of the adsorbed dye. For instance, toluidine blue stains the nucleic acid, in the nucleus or in the cytoplasm, blue, whereas it stains the basophilic granulae of certain leucocytes ("mast cells") purple. This phenomenon, one single dyestuff staining different histological elements in different shades, was called by Ehrlich metachromasy. This phenomenon must not be confused with "differential, or selective" staining, when the tissue is exposed to a mixture of two dyes, and different histological elements adsorb selectively the one or the other dye. Those histological elements which stain metachromatically may be called chromotropic substances.

Ehrlich used especially thionine as a metachromatic dye, and, of the chromotropic substances, he knew the basophilic granulae of the "mast cells," cartilage, amyloid substance, and mucus. The most convenient metachromatic dye used today is toluidine blue. The nature of the metachromatically staining substances has been recognized by Lison as follows: All of them are, at least as far as they are encountered in histological material, half-esters of sulfuric acid with a colloidal carbohydrate of high molecular weight, sometimes combined with a protein, sometimes not. Such substances are chondroitin-sulfuric acid and cartilage, mucoitin sulfuric acid and mucus, the basophilic mast cell granulae which have been recognized as identical with heparin, which according to Jörpes (1939) is also a sulfuric ester of a colloidal carbohydrate, and several vegetable colloids, among which agar is best known. It is, according to Neuberg (1921) a sulfuric ester of a highly colloidal pentosane in the form of its calcium salt. Whereas all nuclei stain blue with toluidine blue, those other substances stain more or less purple to red, according to conditions. We may, for the sake of brevity, call the color of the stained nucleus the "normal" color of the dye, and the color of those other substrates the "metachromatic" color.

In order to understand the specific behavior of nucleic acid toward basic dyes, we have to discuss the optical properties of those dyes, first in their aqueous solution, and second, when they are adsorbed by negatively charged colloids other than nucleic acid. Only then will the contrast in the behavior of nucleic acid become obvious.

The Optical Properties of Basic Dyes in Solution

In most cases, the absorption spectrum of a dyestuff is characterized by a rather sharp absorption band in the visible, and usually there is another sharp band in the quartz-ultraviolet, which in some instances (methylene blue) is almost as strong as the visible band, in other cases much weaker. All the variations to be discussed here are concerned with the band in the visible spectrum. The band in the ultraviolet is much less subject to variation of conditions and furthermore, in admixture with nucleic acid, is difficult to observe because nucleic acids themselves absorb strongly in the ultraviolet. For our purpose we neglect entirely the ultraviolet band.

The absorption spectrum of a dyestuff is not definitively determined by its chemical structure alone but depends also on the environment, such as the nature of the solvent, its concentration, the nature of the substrate by which it is absorbed, and in this case sometimes also considerably, the temperature. I am not speaking of the influence of pH

at all, because this well-known influence depends on a change of chemical structure, namely, on electrolytic dissociation into ions. We shall consider here the change of the absorption spectrum by influences other than pH, and shall consider only such dyes as do not respond to changes of pH within that pH range in which one has to work. Let us start with the absorption spectrum of a dye in a very dilute aqueous solution, at a concentration of about 10^{-6} to 10^{-5} M. A generalized description will not fit all dyestuffs in every detail. The following characteristics hold especially for the dyestuffs of the thiazine and oxazine classes, in which the distinction between normal and metachromatic staining is especially well developed. If we measure the extinction for monochromatic light of varied wave lengths and plot the extinction coefficients, divided by the concentration of the dye, for a light path of 1 cm. in the absorption cell, or the "molar extinction coefficient," against wave lengths, there is usually a rather sharp absorption band in the visible, the maximum of which is characteristic of each individual dyestuff both with respect to its absolute value and to its wavelength. The absolute value of the maximum does not vary very much among "good" dyestuffs; it is usually between 60,000 and 100,000. The band declines steeply toward longer wavelengths, and somewhat more gradually toward shorter wavelengths, usually in such a manner that there is a very slight hump or shoulder, something like 30 mμ apart from the maximum for most of the basic dyes that may be used in histological staining.

On investigating the dye in solutions of higher concentrations one may distinguish two classes of dyestuffs, which may be distinguished as the "polymerizing dyes" and the "non-polymerizing dyes." The distinction is probably not sharp; there are intermediate cases, but for the present purpose we may disregard the intermediate cases. In one class the molar absorption curve is independent of the concentration up to such concentrations as are accessible to accurate optical measurements, which are restricted by the fact that for high concentration one has to use very narrow absorption cells. Below say 0.005 cm. it becomes almost impossible to calibrate the thickness of an absorption cell precisely. But, as far as it it possible to say, the molar absorption curve of some dyestuffs is independent of the concentration. Such substances are said to obey Beer's law. There are relatively few dyestuffs of this type: phenosafranin, trypoflavine, malachite green, methyl green.[1] However, in most of the basic dyestuffs this law is not obeyed; rather is the peak of the molar extinction curve lowered with the increase of concentration, and at the expense of this loss of optical density at this absorption maximum a second band arises, usually about 30 mμ or so from the main band, seemingly at the same wave length where the little hump of the curve in high dilution appears. The higher the concentration, the lower is the first, or α-, band, and the higher the "β" band. This holds for aqueous solutions. In alcoholic solution the peak of the α band is usually somewhat higher than in water and there is no change on increasing the concentration; there is no β-band in alcohol (Beer's law is obeyed). This is the main reason why a solution of, say, methylene blue, has a different shade in water and in alcohol, although a slight displacement of the α band according to the nature of the solvent also contributes to that fact.

As to the interpretation of the β band, there seems to be general agreement, at least where the widely used thiazine and oxazine dyes are concerned. One cannot be sure that the following statements are correct also for all other classes of dyestuffs. Especially I wish in this paper to avoid all references to the triphenylmethene dyes (fuchsin, crystal violet) which would require a special discussion of no interest in this connection; and moreover have not been studied sufficiently in this respect. They present special difficulties. Restricting ourselves to the dyestuffs of the thiazine class, the following statement may be made.

The α band is characteristic of the single molecules of the dye, the β band is characteristic of a bimolecular aggregate of the dye. The cation of the dye dimerizes, reversibly, the equilibrium being determined by the reaction: 2 $M^+ \rightleftharpoons D^{++}$ where M^+ is the positively charged (monomeric) cation of the basic dye, and D^{++} is the dimeric compound, with two positive charges. The equilibrium is determined by the equation:

$$[M^+]^2/[D_n^{++}] = K$$

Although E. Rabinowitsch and Epstein (1941) seemingly obtained a constant value of K on spectrophotometric analysis in solutions of different concentration both for thionine and methylene blue, other authors were less satisfied with experimental verification of the equation. There are various reasons why this might be so. Without discussing all of them, one reason may be that the polymerisation is not restricted to dimerization, but in higher concentration higher polymers may also be formed. The formation of higher polymers can be recognized spectrophotometrically by the fact that the higher polymers show an absorption band still farther displaced toward shorter wave lengths. We may call such a displaced band a γ band, although there is probably, according to the degree of polymerization, not one distinct γ band, but a series of overlapping bands. A γ band may appear in an aqueous solution of sufficiently high concentration. Such a γ band can also readily be pro-

[1] It may be added that the bivalent cations of other basic dyes, say, methylene blue, or toluidine blue, and many others, as they exist when dissolved in very strongly acid solutions, also obey Beer's law.

duced in a highly dilute solution of a dye such as methylene blue or toluidine blue by adding a neutral salt such as ammonium sulfate to the solution. According to the concentration and the nature of the salt, the dye may be "salted out" or may stay in a relatively stable colloidal solution, the particles consisting of clusters of molecules with not much tendency to precipitate. Such a colloidal solution of a polymerizable dye shows the "γ band" (Fig. 1). So we have a definite band characteristic of the

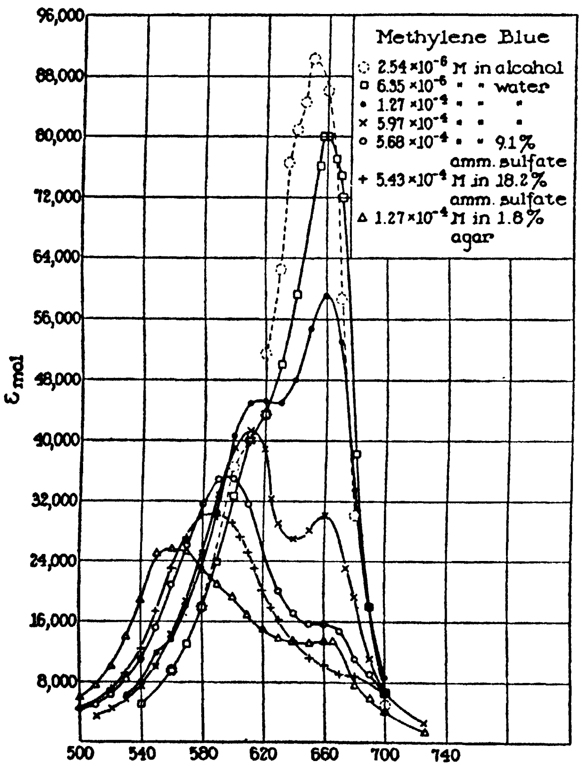


FIG. 1.

monomer (α band), another for the dimer (β band), another, or a group of closely overlapping bands, for higher molecular aggregates (γ band). The equilibrium between the monomers and the di- and polymers is also shifted by change of temperature in a reversible manner. Increase of temperature always favors the establishment of the monomer.

Such dyes which show no β or γ band, such as phenosafranine, are influenced by addition of neutral salts only insofar as the whole extinction curve is depressed, but not displaced. The depression is understandable. When the dye molecules are present in clusters, instead of being homogeneously molecularly dispersed, more light is transmitted across the absorption cell, and the extinction curve is lowered. On the other hand, the establishment of α, β or γ bands in the polymerizing dyes is not so readily understandable. All we can say is that the energy

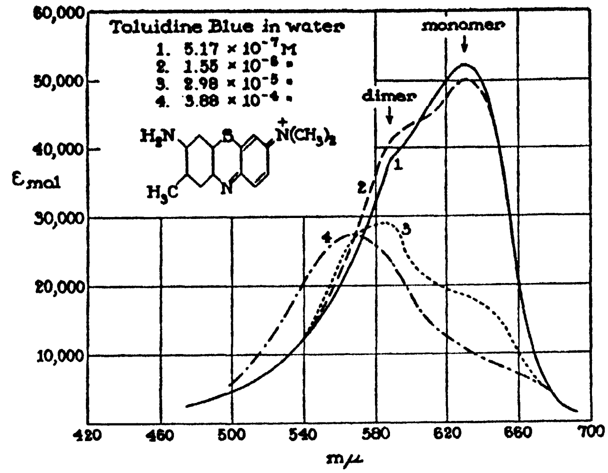


FIG. 2.

quantitation of the molecule is not the same in the monomer as in the di- or polymer, or that the probabilities of transition from one energy level to another are altered on polymerization. In general, dyestuffs obeying Beer's law in aqueous solution do not readily form a stable colloidal solution on the addition of a proper amount of a neutral salt, but instead, as soon as the salting-out effect of a neutral salt starts, the dyestuff has a great tendency to precipitate from the solution in crystalline form. On the other hand, a dyestuff such as methylene blue, in a sufficiently dilute solution, may stay in a kind of colloidal solution without crystallizing after addition of, say, ammonium sulfate in a high concentration, with characteristic optical properties.

THE OPTICAL PROPERTIES OF BASIC DYES IN THE ADSORBED STATE

Proceeding now to the absorption spectrum of dyestuffs when they are adsorbed on a stainable substrate, one has to distinguish two kinds of changes as compared with the spectrum in an aqueous solution. In the first place, the absorption maximum may be somewhat changed both as to its height

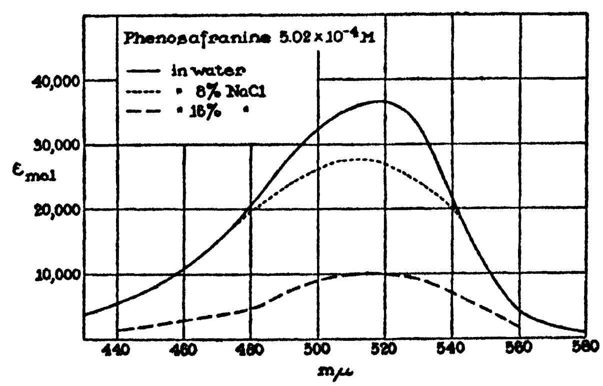


FIG. 3.

and its location. Such a displacement has been used in the study of dyes, especially acidic dyes, by protein solutions. Just as the extinction maximum may be somewhat different according to the solvent in which the dyestuff is dissolved, so it may also depend on the nature of the substrate by which it may be adsorbed. Secondly, and this is more important here, the relative heights of the α, β, and γ bands are altered. For instance, a dyestuff at a given concentration may show almost only an α band in aqueous solution, but when adsorbed may show almost only the γ band; or, it may show in aqueous solution a weak α band and a strong β

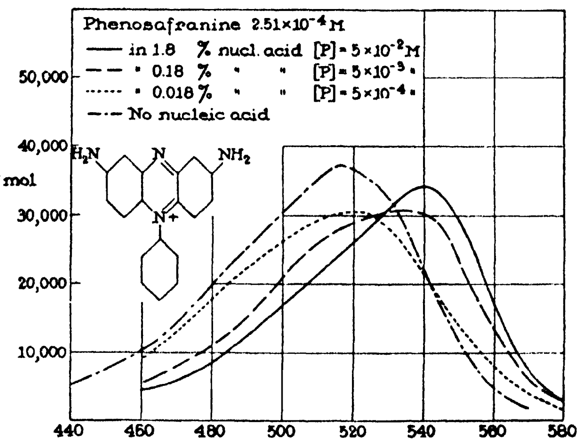


FIG. 4.

band, but at the same concentration, when adsorbed by a colloidal solution of a stainable substrate, may show a strong α band only. It is essentially the shift of the relative heights of the α, the β and the γ bands, due to the adsorption of the stainable substrate that will be considered in what follows.

Non-polymerizing dyestuffs (phenosafranin)

In a non-polymerizing dye, the only difference in the absorption spectrum according to whether the dye is dissolved in water, or alcohol, or adsorbed by any stainable substrate, consists of a usually slight difference of the wavelength of the extinction maximum and, especially, the absolute height of this maximum. Fig. 4 shows this for phenosafranine adsorption by nucleic acid. Fig. 3 shows, as the effect of the addition of salt, only a depression of the molar extinction curve. Fig. 4 shows especially that adsorption of the dye by a solution of yeast nucleic acid just displaces the maximum, more or less according to the ratio of dye to nucleic acid, without any secondary band ever arising.

Polymerizing dyestuffs

The polymerizing dyes are much more interesting. In the following, the behavior of toluidine blue will be demonstrated as an example. Fig. 2 shows the molar extinction curve of its aqueous solution in different concentrations. In extremely dilute solution there is the monomeric, or α band at 630 mμ. As the concentration increases the α band is more and more depressed, and a new absorption maximum arises, the β band, at 590 mμ. At the highest concentration the α band has entirely disappeared and the β band seems even somewhat farther displaced, indicating that probably a higher molecular aggregation has occurred also to a slight extent.

What happens when this dye is adsorbed will be shown first by using a solution of sodium oleate

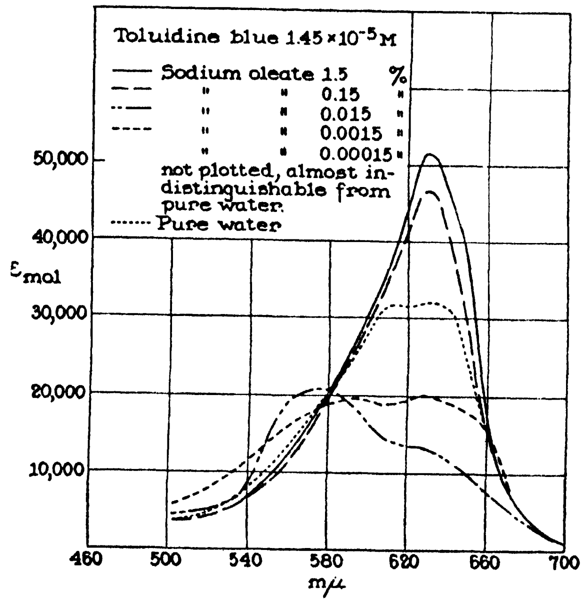


FIG. 5.

as adsorbent. Fig. 5 shows absorption curves of toluidine blue, all for a concentration of 1.45×10^{-5} M, in which for a pure aqueous solution not only the α, but a strong β band is already developed. The effects described here for sodium oleate are exhibited in a similar fashion by all detergents consisting of a long hydrocarbon chain with an anionic group at one end, either a carboxyl, or sulfate, or sulfonate group.

When the soap is in large excess over the dye, the α band is increased, the β band entirely disappears. The soap "depolymerizes" the dye. The interpretation is this. Only a few of the carboxyl groups are combined with a cation of the dye. Since each carboxyl group binds only one dye cation, the dimerization of the dye which would occur in the absence of soap is counteracted and the adsorbed dye appears entirely in the monomeric form. Since only a few carboxylic negatively charged groups are neutralized, the electric repulsion of the soap micelles is still sufficient to maintain a macrohomogeneous colloidal solution. This is no longer

true when the concentration of the soap is lowered, that of the dye being kept constant. Coagulation may occur, with the formation of larger, stained soap micelles, and even a flocculation. In the same degree the α band (630 mμ) is diminished, a β band (590 mμ) appears and finally even an approach to a γ band at 575 mμ is formed. Then, when the concentration of soap is still further decreased, the dye is no longer completely absorbed, and the absorption curve, of course, approaches that of an aqueous solution of the dye. Accordingly, with decreasing concentration the visible color varies

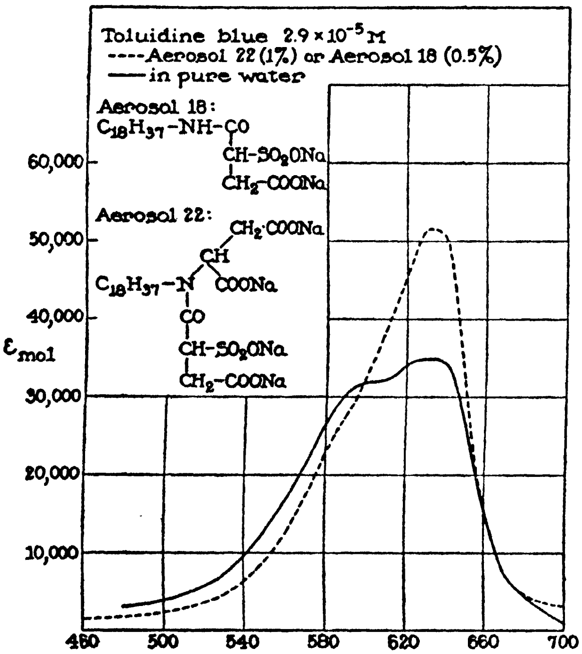


FIG. 6.

from blue to violet to pink-purple. So we may say that sodium oleate according to its concentration relative to that of the dye is stained either in the normal or in metachromatic color.

Another example of an adsorbent, shown in Figs. 6 and 7, is Aerosol 22, which also contains a long hydrophobic hydrocarbon chain and several negatively charged, hydrophilic groups attached to it. The concentration of the dye in Fig. 7 is chosen so that in pure water the β band is already stronger than the α band. When the colloid is applied in high excess, the α band is strongly increased and the β band has almost vanished. The dye is "depolymerized." As the concentration of the colloid is decreased, the α band goes down, the β band goes up, and finally only a strong γ band at about 580 to 590 mμ remains. In Fig. 6 a lower concentration of dye is used, so that in aqueous solution the α band is higher than the β band. When the dye is dissolved in the colloid solution of high concentration, the α band increases, the β band disappears. The interpretation is the same as with sodium oleate.

A further example of a stainable colloid is carboxy methyl cellulose. This colloid differs from those discussed above by the fact that it contains in addition to the acidic side chains, namely, the carboxyl group, many more hydrophilic hydroxyl groups and has no long hydrophobic hydrocarbon chain. It is likely that the difference of its behavior may somehow be correlated to this difference in structure. The difference with respect to its behavior toward

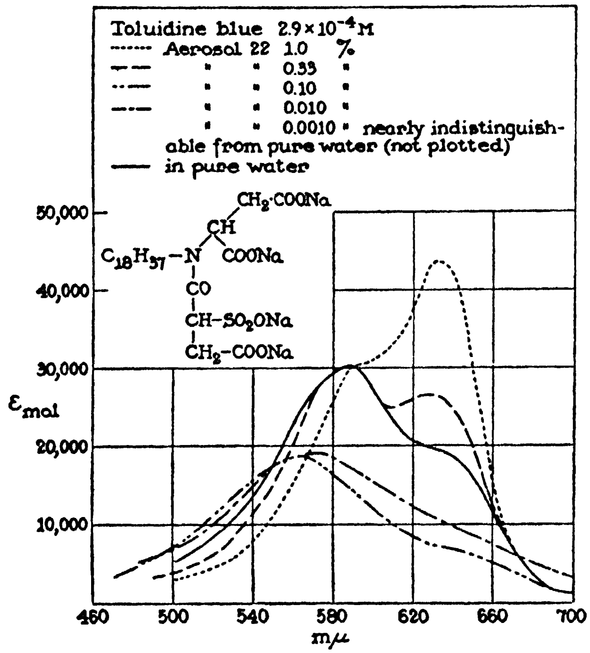


FIG. 7.

the dye is this. Whereas for the other colloids a large excess of the colloid brings about a complete depolymerization of the dye, leaving only an increased α band, no depolymerization at all of the dye occurs with this substrate. Even with large excess of the colloid, the dye is not depolymerized; on the contrary, it is polymerized to a higher extent. In this case there is no α or β band, but only a γ band, and the same holds for lower concentrations of the colloid, until finally on further dilution of the colloid, the dye is no longer adsorbed and the curve for an aqueous solution of the dye is approached. This colloid is stained metachromatically under all circumstances, whereas for the previous cases, metachromasy depended on the ratio of substrate to dye.

The same phenomenon can be seen even to a much higher extent with agar, a substrate which has been known for a long time to be stained metachromatically (Figs. 9, 10). The concentration of the dye in the experiment of Fig. 10 is so low that

in aqueous solution there is a strong α band (630 mμ) and a weak β band (around 590 mμ). Yet in agar, there is always only a γ band, which lies from 540 to 520 mμ, according to the ratio of agar to dye, indicating that there is certainly not one polymer of definite size, but mixtures of molecular aggregates of various sizes.

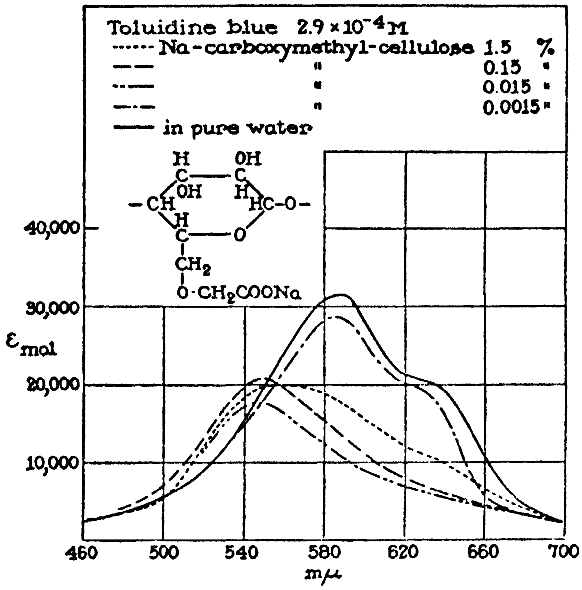


FIG. 8.

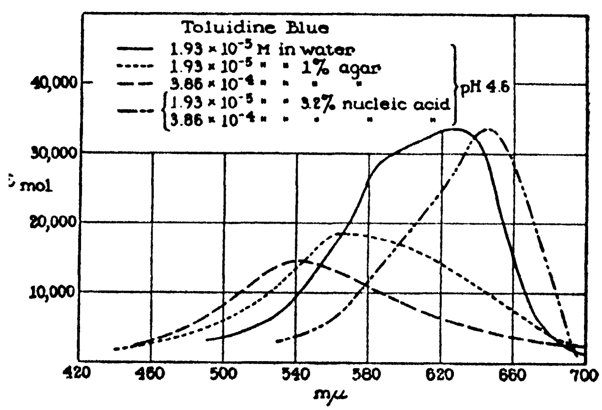


FIG. 9.

These curves are all obtained from optical measurements at room temperature. On heating, the metachromatic red color turns blue, the polymerization is abolished, probably simultaneously with the dissociation of the colored colloid into free colloid and free dye. On cooling, very gradually, the color change is reversed and this process is entirely reversible any number of times. The attainment of the final equilibrium, judged from the change in color, lags somewhat behind the change of temperature, but a definite reproducible equilibrium, depending only on temperature, is reached eventually within 15 to 30 minutes.

To summarize, all the colloidal substrates discussed above may be stained metachromatically; some of them only under certain conditions of relative concentration of dye and colloid; others, meta-

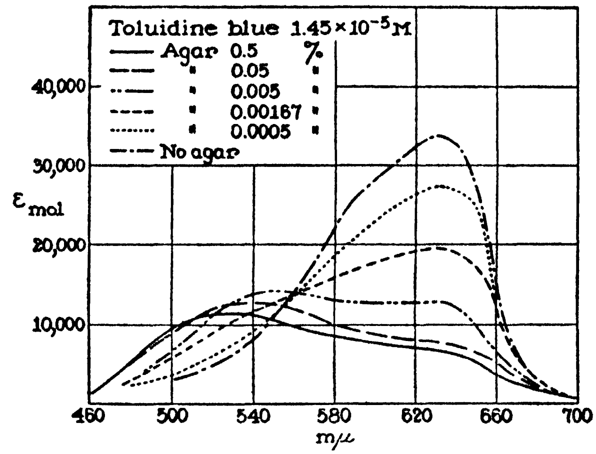


FIG. 10.

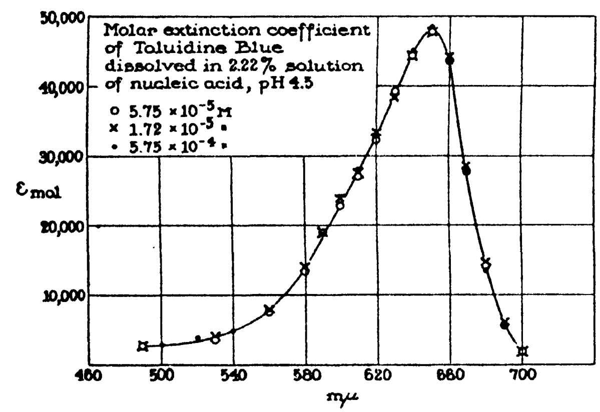


FIG. 11.

chromatically under all conditions of concentration. Nucleic acid, however, does not fit into this scheme.

Basic Dyes and Nucleic Acid

Proceeding now to nucleic acid as stainable substrate, we may start with a clear solution of yeast nucleic acid, dissolved in a dilute buffer so as to establish a pH between 4.5 and 7.0, usually about 5.0. First of all we consider the dye solution at varied concentrations, but always keeping within the range of very dilute dye solutions, the solvent being a highly concentrated solution of nucleic acid (1 to 3%). Fig. 11 shows that under such conditions there appears always only one band, the α band, whereas in aqueous solution as shown before, with increasing concentration the α band decreases

and a β band increases. The dye is vigorously depolymerized by nucleic acid. No new band arises on increasing the dye-concentration, rather does the absorption curve obey Beer's law: it is independent of concentration and is characteristic of a pure monomer of the dye. It is the same situation as with sodium oleate, and in contrast to carboxycellulose or agar. This, however, is true only if the concentration of nucleic acid is very high. We have now to consider what happens in less concentrated solutions of nucleic acid. (The results obtained for this case have not yet been described in my previous paper (1945)). Figs. 12 and 13 show what happens when, at constant dye concentration, the concentration of nucleic acid is varied. In curve 1 (Fig. 12) (aqueous solution) there is an α, and a β band. In 2.55% nucleic acid (curve 6) there is one high α band, no β band. In 0.30% nucleic acid there is only a negligible change; the dye almost obeys Beer's law so far. However, on further decreasing the concentration of nucleic acid to 0.03% (curve 4) the α band is somewhat lowered, and a slight β band appears. On further lowering the concentration of nucleic acid, the α band further decreases, and the β band becomes stronger (curves 2 and 3). Finally, on further lowering of the concentration, the curve of course gradually approaches that for aqueous solution. There is never a condition where a γ band would be formed. The same can be seen

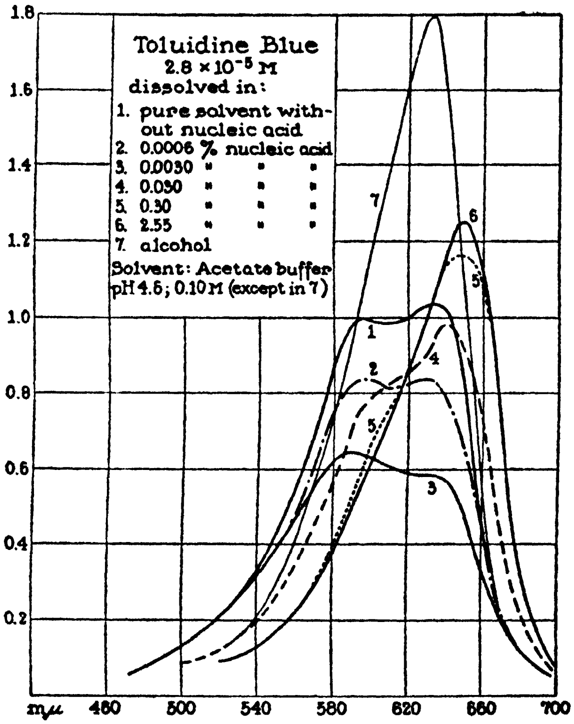


FIG. 12.

in Fig. 13. Curve 3 is for the condition where the concentration of the dye equals approximately that of the phosphoric groups of the nucleic acid. Here we have an α and a β band. As regards the interpretation of those curves in which both bands appear and the absolute height of the bands is lowered, there are two possibilities. One interpretation is that the adsorption of the dye is incomplete and that the β band represents essentially the adsorbed

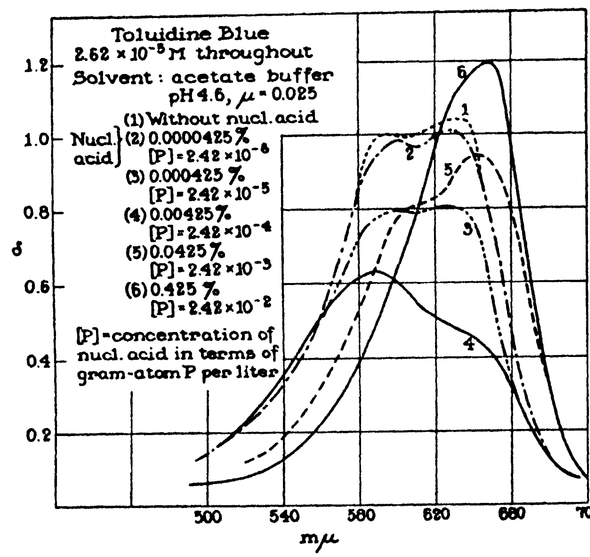


FIG. 13.

dye, the α band the free dye. Another possibility is that the dye is entirely adsorbed and exhibits in the adsorbed state the spectrum of a partially dimeric dye. From the observations on stained tissue slices, to be discussed later, we should prefer the second alternative.

As regards the visible appearance of color in toluidine blue, it makes very little difference to the unaided eye whether or not there is a β band in addition to the α band; in either case the color may be said to be blue, or the "normal" color. However, a γ band shifts the visible color toward purple or even pink. So, nucleic acid is stained always blue by toluidine blue, but agar always pink to violet. The fact that just in toluidine blue the γ band has such a large effect on the visible color renders this dyestuff especially useful. On purely spectrophotometrical observation, methylene blue is just as suitable, but the change of color as seen directly is not so impressive.

It is characteristic for nucleic acid that under no condition does a γ band appear, whereas for other stainable colloids a γ band appears either under all conditions (agar and all histologically occurring metachromatic substances) or under certain conditions of concentration (as in sodium oleate). Fig. 14

shows the effect of nucleic acid on thionine, and shows also that protamine has almost no effect on the extinction curve for thionine except for a slight depression. A quite similar effect of nucleic acid is shown for pyronine (a diphenyl-methane dye). So far, no difference in the behavior of a solution of yeast nucleic acid of low viscosity and low molecular weight and a turbid and viscous solution of thymonucleic acid of high molecular weight could be found.

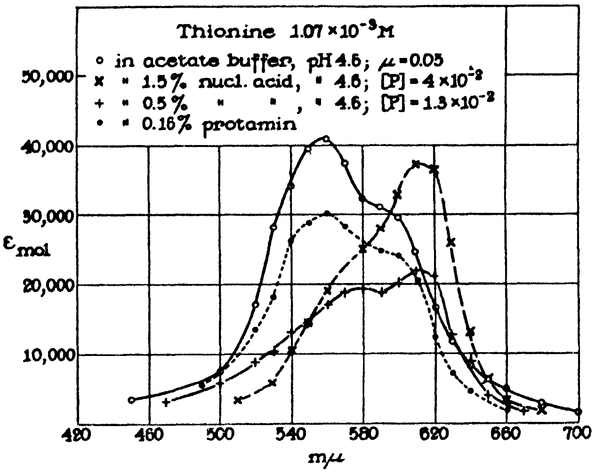


FIG. 14.

Proceeding now to the staining of nucleic acid in a slice of fixed tissue, the conditions are no longer favorable to quantitative measurement of molar extinction coefficients. Such a concept has a meaning only if the concentration of the dye in its medium is known. However, on the basis of experiments just described with *solutions* of the colloidal stainable substrate, a background of general experience has been established which allows of making certain definite statements about the staining of *tissue slices*.

An essential difference between staining of tissue slices and the experiments just described is the fact that in histological staining methods the solution of the dyestuff is always of very much higher concentration than in the previous experiments. Dyestuff concentrations such as were used in the previous experiments are in general too low to bring about satisfactory staining of tissue slices or smears. When stained sufficiently with a more concentrated dye solution of the order of 1% (about 0.03 molar), a slice, say of liver tissue, 20 to 30μ thick, may be easily investigated with a microscope-spectroscope and the analysis of the color with respect to α, β, or γ bands is readily accomplished in a qualitative or semi-quantitative manner. A tissue slice of liver, in which nucleic acid and hence basophily is not restricted to the nucleus but prevails also in the cytoplasm, is stained with aqueous solution of the dye, the excess of dye being washed out first with water, then with alcohol until no dye can be further extracted; the slice is then observed with the microscopic spectroscope. The result depends on the medium in which the observation is made. To make the results comparable to the previous model experiments with solutions, the slice is first observed when mounted in water. A very distinct β band, for instance, for toluidine blue at 590 mμ is visible, and no, or only a very weak α band (630 mμ) ap-

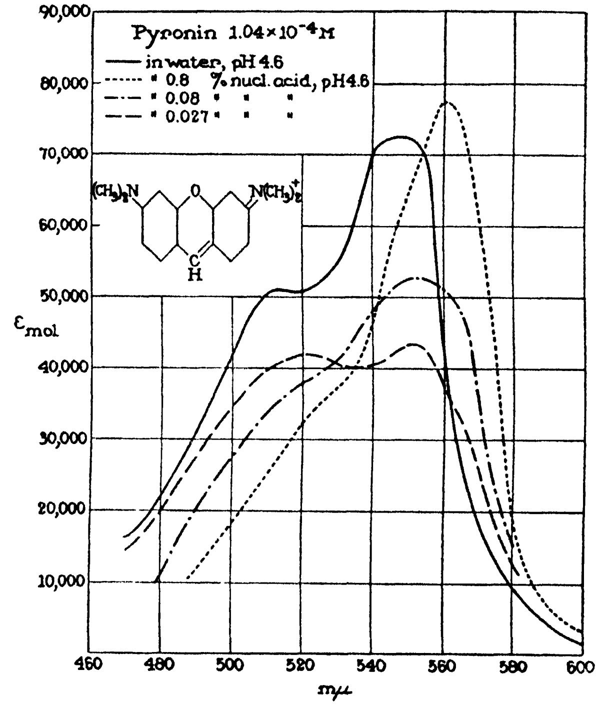


FIG. 15.

pears. The result is analogous to other thiazine dyes such as methylene blue, thionine, but also to some triphenylmethane dyes, especially crystal violet (of course not with methyl green or phenosafranin, which never develop a *β* band). There is never, in spite of the high concentration of the dye, a γ band, as would appear in metachromatic staining. When now the same slice is mounted in alcohol, the β band disappears and a pure and strong α band arises. This shift is reversible; changing from water to alcohol and vice versa invariably brings about the same shift of absorption bands. The unaided eye may just distinguish a slight difference in shade: in alcohol it is blue, in water it has the faintest shade of violet in it. On changing from alcohol to xylene and to balsam, no further shift takes place, the pure α band remains.

The result in water is that expected from the above model experiments on nucleic acid. Since the concentration of dye is high, one expects the β

band, and it is in agreement with the model experiments that a γ band never develops. In spite of the high concentration of the dye, which stains a thin microtome slice intensely blue, no γ band is developed. The shift from water to alcohol has no analogue in the model experiments in solutions: nucleic acid is not soluble in alcohol.

The absence of a γ band under any conditions and the appearance of the β band, which in an alcohol medium shifts, reversibly, to an α band, may be said to be a characteristic property of stained nucleic acid in histological preparations. This property has not been observed for any other stainable substrate. The mere description of this fact may be worth while. It will be more interesting if one should succeed in its interpretation and possible evaluation for the knowledge of the structure of nucleic acid. This staining effect may quite generally be interpreted as the tendency of nucleic acid to counteract the polymerization of the dye, in contrast to metachromatically staining substrates, which show the tendency to enhance polymerization of the dye. It is a justifiable and not far-fetched speculation that this specific property of nucleic acid is correlated with its structure. Nucleic acid whether of high or low molecular weight, may be imagined to consist of strings or bundles of nucleotides arranged in such a way that the pyridine, or purine rings lie parallel to each other, connected by phosphate groups; the dye molecules attached to the negatively charged end of the phosphate group. Each dye cation combined with one phosphate group must lie in the space between the planes of the pyridine or purine rings, and so they are prevented from approaching each other in such a way as to interfere optically with each other and from exhibiting the spectrum of a higher dyestuff aggregate. The fact that alcohol reversibly changes the β band into an α band, needs some comment. This problem is correlated with the problem of why polymerization influences the absorption spectrum at all. It seems to be generally true that in such dyestuffs as are capable of developing a β band, such a β band always develops when dyestuff molecules are forced to lie close together in pairs, and a γ band, when the dye molecules form still larger clusters. The close approach of two dye molecules may be brought about in two ways: either spontaneously, on increasing the concentration whereby dimeric molecules are formed, or by the addition of a neutral salt bringing about polymerization by an incipient salting out effect. I do not wish to account for the forces responsible for holding together the molecules at this occasion, since the theory is still very uncertain. However, it is understandable that the spectroscopic properties of the molecule may be changed on dimerisation. As mentioned before, the β band arises at the same wave length where the α band has a very slight hump. So it is probable, as Sheppard has suggested, that the β band is not a new band but is present, although to a slight extent, in the spectrum of the monomer in the form of that slight hump, and that on dimerization the intensity of the hump increases, producing a β band. Sheppard also pointed out that the dimeric or polymeric spectrum appears only in aqueous solution, not in alcohol or other organic solvents. He assumes that a water molecule lies between the two dyestuff molecules, held by a kind of hydrogen bond, and that this establishes the bonding of the two dye molecules. The effect of alcohol would then be to prevent the water molecule from being captured between the two dye molecules, and so at the same time preventing the dimerization and any change of the absorption spectrum on increasing the concentration of the dye. In a fixed tissue it is unlikely that changing from an aqueous to an alcohol embedding medium would change the location of the dye molecules with respect to the phosphoric group or to each other. Here we may say, when two dye molecules which have just space enough to squeeze into the space between two pyridine, or purine rings, are separated by water molecules, the dimeric spectrum will develop. When, however, alcohol withdraws the water, the monomeric spectrum is established.

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DISCUSSION

FRIEDENWALD: It may be of interest to report that the distinction between the metachromatic and the orthochromatic staining of tissue components can be greatly exaggerated by staining in the presence of high concentrations of magnesium. Using methylene blue, and staining at pH about 1.5, the nuclei almost completely fail to be stained if the staining solution is saturated with $MgCl_2$ while cartilage and mast cell granules still stain with normal intensity. I have assumed that this may be due to a blocking of the phosphate groups by magnesium.

CHARGRAFF: I was very much interested in Dr. Michaelis's remarks about the nonstainability of living nuclei. If we really assume that in the nucleus the nucleic acid is bound to a basic protein such as histone by electrostatic bonds, *i.e.,* that nucleohistone is a histone nucleate, then the simplest explanation is that given by Dr. Michaelis, namely that the basic dye displaces the histone. But I do not know whether the evidence available at present really supports the view that the nucleic acid-protein bond is mediated through the phosphoric acid groups. It is conceivable that in an undegraded nucleoprotein the phosphoric acid groups are available for combination with basic dyes. Recent experience in our laboratory with nucleoprotein fractions of tubercle bacilli and their precipitability by lanthanum salts would seem to favor this possibility.

KURNICK: Dr. Michaelis has aptly demonstrated for us the basis of the metachromatism manifested by many basic dyes. We have been interested in another problem in staining by basic dyes.

The remarkable ability of pyronin to distinguish between desoxyribonucleo- and ribosenucleo-proteins in tissues, as evidenced, for example, by the staining of myeloma cells (from plasmacytoma) when pyronin is used alone or in the mixtures of Unna and Pappenheim (see also Dr. Ris's paper), has been a puzzling phenomenon. This basic stain is apparently capable of staining one acid material (ribosenucleic acid) but not another (desoxyribosenucleic acid).

We have been interested in determining whether or not this stain is capable of similar distinctions under *in vitro* conditions in which the two nucleic acids are handled under identical conditions. We have found that staining precipitated nucleic acids with aqueous pyronin, washing thoroughly with acid-alcohol, and then dissolving the entire stained precipitate in concentrated HCl and comparing the solutions spectrophotometrically, gives reproducible results. Under these conditions, highly polymerized desoxyribonucleic acid stains about one-fifth as intensely as ribonucleic acid; desoxyribonucleohistone stains about one-sixth as intensely as ribonucleoprotein (all comparisons are made on the basis of equal weights of nucleic acid phosphorus); desoxyribonucleohistone stains about two-thirds as intensely as *highly* polymerized desoxyribonucleic acid; and ribonucleoprotein stains with approximately the same intensity as ribonucleic acid. Desoxyribonucleic acid, however, which has been considerably *depolymerized,* stains with the *same intensity* as ribonucleic acid. In other words, under the conditions used (with acid alcohol as the "differentiating" wash), pyronin distinguished *not* between desoxyribonucleic acid and ribonucleic acid, but between highly polymerized and depolymerized nucleic acids. The distinction, in these experiments is between *states of polymerization* of nucleic acids rather than between the chemical differences of the nucleic acids studied. It is possible that the uncovering of dibasic phosphoric acid groups, which occurs during depolymerization, accounts for the more intense staining of the less highly polymerized acids. Differences in physical states between high polymers and low polymers may also play a role, since pyronin and methyl green are easily separated from mixtures by paper or permutit (Decalso) adsorbtion columns.

When drops of the solutions used above are placed on glass slides (coated with Mayer's albumen) so that each drop contains the same amount of nucleic acid phosphorus (.0025 mg P in .01-.025 cc was used), allowed to dry, stained with Unna's pyronin methyl green mixture and differentiated in 95% alcohol (acid alcohol is too rapid, removing practically all visible stain, just as it does with tissue sections), the ribonucleic acid stains pale pink, the depolymerized desoxyribonucleic acid almost identically (there may be a trace of purple rather than a pure pink), the polymerized desoxyribonucleic acid stains green, exactly as does the nucleohistone, desoxyribonucleohistone a deep pure green (visually), and ribonucleoprotein faint pink with a trace of purple (identical to the depolymerized DNA).

One may conclude, therefore, that pyronin distinguishes only between states of polymerization of the nucleic acids if either alcohol or acid alcohol are used to wash. The choice of wash fluid depends upon the mass of nucleic acid to be "differentiated"; acid alcohol is the choice when the mass is large, but is too fast for small amounts, while alcohol is the choice for low concentrations, but too slow for high concentrations.

Furthermore, it is apparent that methyl green behaves in a manner opposite to that of pyronin, adhering more firmly to the higher polymers. Further quantitative experiments remain to be done.

Since desoxyribonucleic acid usually occurs in cells in a very highly polymerized form, and ribosenucleic acid is a relatively low polymer, it appears probable that the apparent distinction between

nucleic acids in tissues stained with pyronin is a function of their relative states of polymerization.

MICHAELIS: Dr. Kurnick's findings are very interesting and very important with respect to the interpretation of the staining properties of the methyl green-pyronin mixture discovered about 50 years ago by Pappenheim. This staining method depends on selective staining, in so far as different stainable substrates, when exposed to a mixture of two dyestuffs, adsorb preferably one or the other of the two dyes. My own experiments, however, deal only with staining with a solution of a single dyestuff. They point out the difference in behavior of any nucleic acid as compared with that of any other basophilic substrate that may occur in cytological or histological research.

With respect to the problem as to the nature of the metachromatically staining substrates, it has been pointed out in my paper that, according to Lison, all of the metachromatically staining substrates are supposed to be sulfuric esters of high-polymeric carbohydrates. This statement requires an amendment according to what we know now. Not only sulfuric esters, but also high-polymeric carbohydrates with carboxyl groups, such as the capsular substance of pneumococci, are stained, more or less, metachromatically. Furthermore, it has been shown by Wiame that polymeric metaphosphate also stains metachromatically (purple) with toluidine blue. Dr. Wiame sent me smears of yeast cells stained with toluidine blue, which I herewith project on the screen. When the cell contains no metaphosphate, the color is blue, owing to the presence of ribonucleic acid. When the cells, under the same conditions as studied by Wiame, have stored metaphosphate, the staining is violet to purple. For a further detail with respect to the distinction between normal and metachromatic staining, I refer to my answer to Dr. Gulick's question during this Symposium.

GULICK: Have you studied how the staining of mucoitin sulphuric acid, or of mucin cells, by toluidine blue compares with staining of nucleoproteins? It is my impression that the comparison is of considerable interest.

MICHAELIS: On using a metachromatic dye such as toluidine blue, nucleic acid can always be readily distinguished from mucin. Nucleic acid stains blue, whereas mucin stains purple or violet. This is true when the object is mounted in water. It is very often also true when the object is mounted in alcohol or canada balsam. However, some of the metachromatically staining materials, especially mucin, may change their metachromatic color into the normal (or "orthochromatic") one when mounted in alcohol. For this reason it is safer to mount the object in water if one wants to decide whether a substance stains metachromatically or orthochromatically. With these precautions in mind, there is no danger of confusing nucleic acid with mucin.

CHEMICAL PROPERTIES OF ISOLATED CHROMOSOMES

A. E. MIRSKY

In previous investigations of the chemical composition of chromosomes, materials were extracted from cells and tissues, and evidence was presented to show that the materials extracted were indeed derived from chromosomes (Mirsky and Pollister, 1946). While extracting chromosomal constituents from the nucleated erythrocytes of the salmon, chromatin threads were isolated (Mirsky and Pollister, 1943a). Microscopic examination of these threads have shown that they are in fact isolated chromosomes (Mirsky and Ris, 1947). Isolation of chromosomes from nucleated erythrocytes was readily accomplished because practically the whole cytoplasm of these cells is soluble in saline. Chromosomes have subsequently been isolated from the thymus, liver, kidney, salivary gland and other mammalian tissues. From these tissues practically the whole mass of chromosomes present can be isolated, so that in a single preparation as much as 6 grams (dry weight) of chromosomes can be had. All of the work on chromosome chemistry to be presented in this paper has been done on isolated chromosomes.

The first step in preparing chromosomes is to break up the cells in which they lie embedded. For this purpose both a Waring mixer and a colloid mill are used. In this and in all subsequent operations precautions are taken so that temperatures do not rise above 3 or 4° C. Separation of chromosomes from other cellular constituents is accomplished by straining through fine cloth and by differential centrifugation. Straining holds back particles larger than chromosomes, permitting the chromosomes to pass through; centrifugation throws down chromosomes, leaving lighter particles in the supernatant.

It might be thought that it would be advantageous first to isolate nuclei by the use of citric acid or some other weak acid and then break up the nuclei. One reason for not following such a procedure is that once nuclei have been treated with citric acid, it is exceedingly difficult to break them, and the chromosomes then set free are badly deformed. Another objection to the use of citric acid is that it inactivates some of the enzymes present in chromosomes.

To determine how clean a chromosome preparation is, the suspension is stained and examined microscopically. Little or no material that does not stain with aceto-orcein should be present. A more rigorous test is to stain by the Feulgen procedure and then counter-stain with fast green, or to stain with a mixture of aceto-orcein and fast green. Non-chromosomal material will appear green in these tests. Another test is to stain the suspension with the Millon reagent and then observe microscopically whether any bodies other than chromosomes are present. When examined in these ways, chromosomes isolated from thymus lymphocytes are found to be exceedingly clean, those from liver or kidney somewhat less so. These tests would not demonstrate material adsorbed on the chromosomes. A test for adsorbed serum proteins is to determine whether antibodies to these proteins are produced when isolated chromosomes are injected into animals. Antibodies to beef serum are not detectable in the serum of rabbits after injections of beef chromosomes. But using a far more sensitive test, anaphylaxis in the guinea pig, it may be shown that isolated chromosomes are contaminated with traces of adsorbed serum proteins. Tests for the purity of isolated chromosomes show, then, that thymus chromosomes are remarkably free of non-chromosomal particulate matter, but that traces of soluble material may adhere to them.

Since it is of interest to consider the chemical composition of chromosomes in relation to their general morphological features, it is important to know whether the chromatin threads that have been prepared are indeed chromosomes and not merely unspecific nucleoprotein fibres. The evidence that they are chromosomes may be summarized: Like chromosomes they show a definite organization along their axis, such as primary and secondary constrictions, trabants, heterochromatic and euchromatic sections; like chromosomes these threads can be uncoiled by certain agents, KCN for example; chromosomes consist of at least two chromonemata coiled together, and the doubleness of these bodies can usually be seen clearly; they vary greatly in size and organization; and the final proof that these bodies are chromosomes is the repeated occurrence of one and the same type of chromosome in preparations made from the same type of cells.

Before investigations on isolated chromosomes were begun, three components of chromosomes were recognized: desoxyribose nucleic acid, histone and a non-histone tryptophane-containing protein (TrPr) (Mirsky and Pollister, 1946). These three constituents are also found in isolated chromosomes. The first problem is to determine how much of each is present and how they are organized to form a

chromosome. This problem has been studied most thoroughly in the chromosomes isolated from thymus lymphocytes (Mirsky and Ris, 1947). These chromosomes contain 37% of desoxynucleic acid. By treating them with a strong acid, 40% of histone can be isolated, but it will be seen that they actually contain more histone than this would imply. To study the organization of the chromosome, fractionation with M NaCl has proven to be an effective procedure. When isolated lymphocyte chromosomes are placed in M NaCl they at once disperse to form an opalescent, highly viscous suspension. Much material passes into solution, but when a drop of the fluid is placed under the microscope, it can be seen that not all of the chromosome has been dissolved. The thread-like portion of the chromosome that remains undissolved still looks like a chromosome, although it is a good deal smaller than the original chromosome. It will be referred to as a *residual chromosome*. Residual chromosomes exhibit longitudinal differentiation into thicker, tightly coiled heterochromatic sections and more loosely coiled euchromatic regions. As in whole chromosomes, constrictions are frequently visible.

High speed centrifugation separates the residual chromosomes from the chromosome fraction soluble in M NaCl. It is then found that the residual chromosomes represent only about 10% of the mass of the original chromosome suspension. The soluble fraction of the chromosome (some 90% of its mass) consists almost entirely of nucleohistone, there being but little non-histone protein present in this fraction. What has been called the TrPr is, accordingly, the protein of the residual chromosome.

The residual chromosome, as well as the chromosome fraction soluble in M NaCl, contains nucleoprotein. The residual chromosome of the thymus lymphocyte contains 15% of nucleic acid. Most of the nucleic acid of the residual chromosome is ribose nucleic acid, there being four parts of ribose nucleic to one part of desoxyribose nucleic acid.

The two nucleoprotein fractions of the thymus lymphocyte chromosome differ from each other in a number of respects: both the nucleic acid and protein components of these nucleoproteins are different; nucleohistone is dissociated by M NaCl, whereas the ribose nucleoprotein of the residue is not dissociated by even higher concentrations of salt; in nucleohistone the nucleic acid moiety has elongated molecules and so gives the nucleohistone a fibrous character under certain conditions, but the residual chromosome is fibrous because of its protein moiety, for its fibrous appearance remains unchanged even after removal of all the nucleic acid associated with it.

The contrast between the two fractions of the chromosome is strikingly shown in their staining properties. Commonly used chromatin stains, such as crystal violet, aceto-orcein, hematoxylin and Feulgen are effective because they react with nucleohistone. They also stain the residual chromosome, but only faintly. The methyl green-pyronin mixture which, as Brachet has shown, stains desoxyribose nucleoproteins green and ribonucleoproteins red, stains the whole chromosome a blue green, the nucleohistone green and the residual chromosome red.

Both isolated chromosomes and residual chromosomes show marked instability under certain conditions. Chromosomes are isolated in the cold and in a faintly acid medium, approximately pH 6.3. If the pH is raised to 7.2, and the suspension is brought to room temperature, the chromosomes are unstable. They soon begin to stick to each other, forming larger and larger clumps until, finally, what was a suspension of chromosomes is a fairly solid gel. Observed under the microscope during this transformation, the chromosomes appear more and more moth-eaten, until they finally disappear. At the same time that these microscopic and macroscopic changes occur, there appear dissolved in the fluid surrounding the chromosomes substances derived from the chromosomes. These substances are mainly the partly hydrolyzed ribonucleoprotein constituents of the residual chromosomes. There is but little change in the nucleohistone fraction of the chromosomes.

When residual chromosomes are exposed to the same conditions, they remain intact microscopically, although they stain more and more faintly with pyronin. Chemical determinations show that ribonucleic acid (but not desoxynucleic acid) along with some protein is being split from the residual chromosome. Apparently some of the enzymes responsible for the disintegration of the residual chromosome, while it is still part of the whole chromosome, are removed by the M NaCl used in fractionating the chromosome.

The autolytic changes observed in chromosomes are of interest for several reasons: (1) They show that the residual chromosome is essential for the structure of the whole chromosome. If nucleohistone is removed by M NaCl (and this can also be accomplished by the use of certain enzymes), the structure of the residual chromosome remains, but if the residual chromosome is disintegrated by autolytic processes no structure of microscopic dimensions remains in the gel-like nucleohistone. (2) Some of the changes that occur during autolysis may also take place in the living cell under regulated conditions. The diffusible substances liberated from chromosomes may play a part in interactions within the nucleus and between nucleus and cytoplasm.

Some observations on the phosphatase of the lymphocyte chromosome will be mentioned briefly. All of the alkaline phosphatase of this chromosome is found in the residual chromosome. When the residual chromosome disintegrates in chromosome autolysis, alkaline phosphatase is liberated and found dissolved in the surrounding medium. When

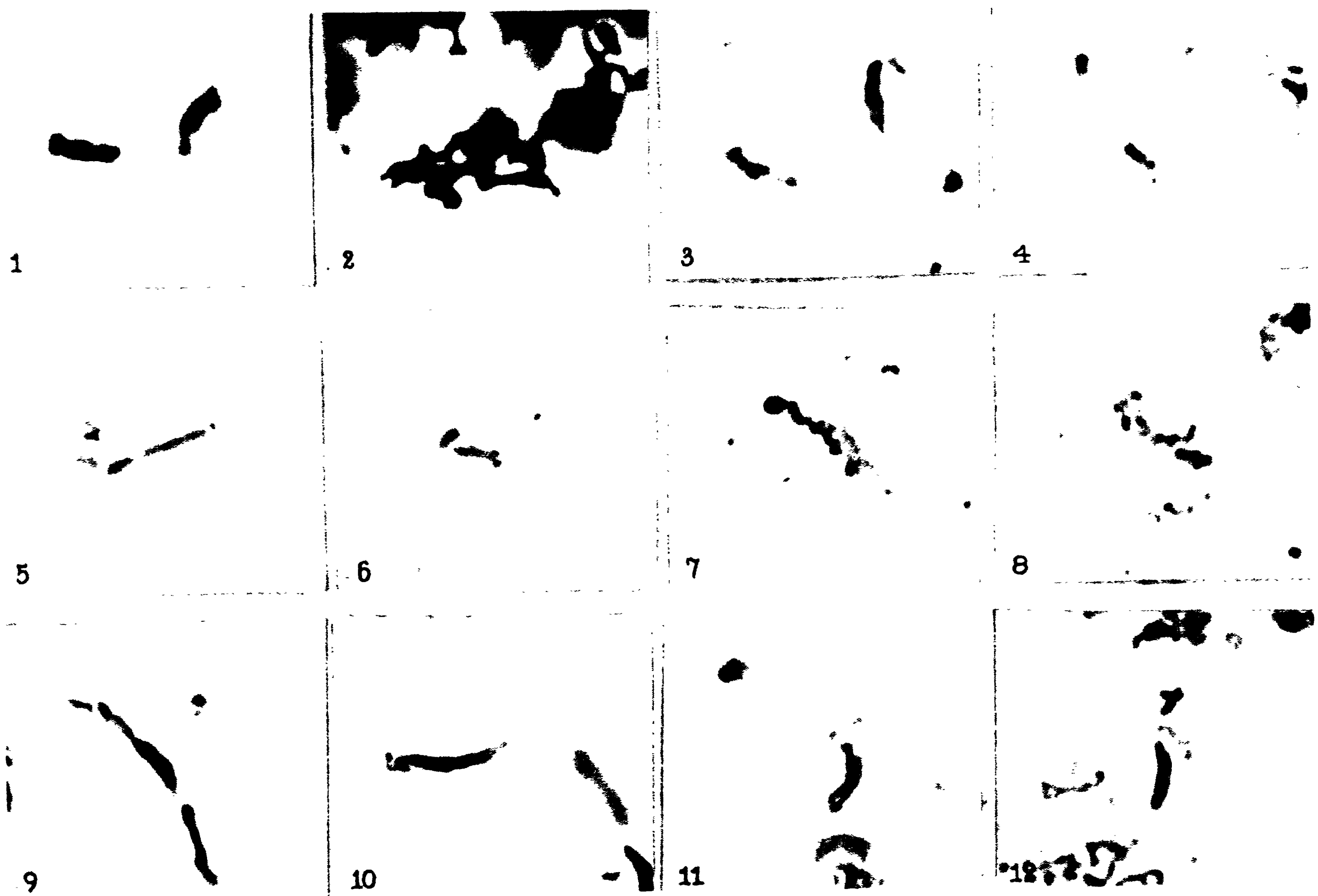
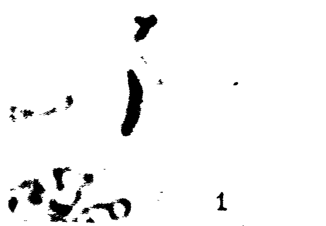


PLATE I

FIGS. 1 and 2. Isolated chromosomes from calf thymus. Aceto-orcein. 3000 ×. FIGS. 3 and 4. Isolated chromosomes from calf liver. Oceto-orcein. 3000 ×. FIGS. 5 and 6. Isolated chromosomes from carp erythrocytes. Aceto-orcein. 3000 ×. FIGS. 7 and 8. Isolated thymus chromosomes, uncoiled with 2.10^{-3} M KCN. Aceto-orcein. 3000 ×. FIGS. 9 and 10. Two identical liver chromosomes from two different preparations. This chromosome can be recognized repeatedly in preparatians of isolated liver chromosomes. Aceto-orcein. 3000 ×. FIGS. 11 and 12. Two identical thymus chromosomes from two different preparations. Aceto-orcein. 3000 ×.



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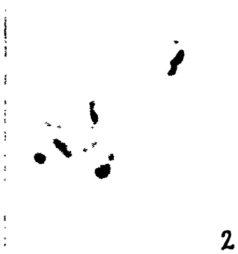

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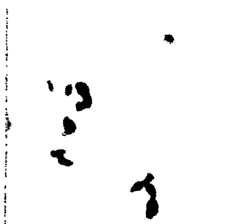

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Fig. 5

Plate II

Magnification of all figures 3000 ×.

Fig. 1. Isolated thymus chromosome. Aceto-orcein. Figs. 2-4. Thymus residual chromosomes, fixed in acetic-alcohol, stained with pyronin
Fig. 5. Suspension of isolated thymus chromosomes. Aceto-orcein. 1200 ×.

ribonucleic acid, along with some protein, is split from residual chromosomes, with the residual chromosome structure remaining otherwise intact, no phosphatase is liberated.

Up to this point the chromosomes described have been isolated from the calf thymus. It is important to consider the chromosomes of other types of cells, for by comparing chromosomes of different cell types, the significance of the component parts of chromosomes will be understood. The chromosomes to be mentioned are those of fish erythrocytes, fish sperm, mammalian thymus, liver, kidney and salivary gland.

A difference between the chromosomes of salmon erythrocytes and salmon (or trout) sperm is that in the former the basic protein linked to desoxynucleic acid is a histone, whereas in the latter it is a protamine (Mirsky and Pollister, 1943b). A similarity in the composition of these two chromosomes is that in both of them the residual chromosome represents an exceedingly small fraction of the whole chromosome. In the fish erythrocyte the residual chromosome is about 5% of the whole chromosome, and in the trout sperm, although precise figures are not yet available, the residual chromosome material is also scanty.

In marked contrast to these chromosomes are those of the liver, kidney and salivary glands. The residual chromosomes of isolated liver chromosomes, for example, represent 40-50% of the mass of the chromosomes, and the remainder is not entirely nucleohistone, for some quantity of non-histone protein is extracted by M NaCl from these chromosomes. Only about 45% of the liver chromosome consists of nucleohistone. This is to be compared with nearly 90% nucleohistone in a lymphocyte chromosome. These figures show that in a liver chromosome there are more than five times as much non-histone protein as in a thymus chromosome. There are also differences in ribonucleic acid content. In a liver chromosome 12% of the total nucleic acid is ribonucleic acid; in a thymus chromosome 3% of the total is ribonucleic acid; and in the trout sperm nucleus only 0.15% of all the nucleic acid is ribonucleic acid.

The cell types, the chromosomes of which have just been compared, may be compared in other respects too. In both a thymus lymphocyte and a trout sperm there is but little cytoplasm; and in a nucleated erythrocyte, although there is a large volume of cytoplasm, this is relatively inactive metabolically. In all these cells the residual chromosome forms an exceedingly small fraction of the whole chromosome. The residual chromosome of liver, kidney, and salivary gland chromosomes, on the other hand, forms close to one-half of the total chromosome; and in these cells the cytoplasm is abundant and active. From these examples it is apparent that a well-developed residual chromosome is associated with a large, active cytoplasm.

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DISCUSSION

BRACHET: In the salivary gland chromosomes of Chironomus, it seems that heterochromatin probably contains more ribonucleic acid than euchromatin. The most remarkable finding, in this material, lies in the fact that a small number of bands stain purely red with methyl green-pyronin; their stainability entirely disappears after treatment with ribonuclease. It is probable that these bands are concerned with the elaboration of micronucleoli.

MIRSKY: We studied the salivary gland chromosomes of Drosophila stained with pyronin-methyl green, but did not find any difference in the staining of euchromatic or heterochromatic regions. It is possible that the different staining of heterochromatic regions in Chironomus is due to the presence of micronucleoli rather than a different composition of the chromonemata themselves.

COHEN: Dr. Mirsky's data present a clear and consistent story. I should like to raise a question concerning the terminology now in vogue, in view of the significance of the dissociating action of M NaCl on nucleohistone. I am referring to the use of the word "chromosin."

When histone and desoxyribonucleate molecules are removed from the chromosome by M NaCl, it is not possible at present to define the position of these individual molecules relative to one another within the chromosome. When the histone nucleate is precipitated in 0.14 M NaCl, it appears probable that the relation of a nucleic acid molecule to a particular histone molecule is not at all the same as their relative positions in the chromosome. Hence the histone nucleate prepared by dissociation in M NaCl and reassociation in 0.14 M NaCl scarcely reflects the positions of the constituents in the chromosome. The word "chromosin" implies a chromosomal relationship which doesn't really seem to exist. In addition, because of its close similarity to the word "chromosomin" applied to another type of material, it does not seem to be a contribution to the nomenclature and can simply be replaced by histone nucleate. I should like to ask Dr. Mirsky and Dr. Pollister if it is not possible to discontinue the use of "chromosin" at this time.

MIRSKY: The term chromosin was used by us in a recent paper because of Bensley's use of the term plasmosin to describe the same material and because of Szent-Györgi's use of the terms hepatosin, renosin, etc., also to describe the same material with

which we have worked. Chromosin at that time expressed better than did plasmosin the location of this material within the cell. Furthermore, our chromosin preparations did not consist entirely of nucleohistone. In the present paper the term chromosin was not used, and this obviously indicates that we have already abandoned the term as we have come to a better understanding of the nature of our "chromosin" preparations. I am glad that Dr. Cohen has raised this question so that we can make our position more explicit than we have.

WEISSMAN: We have isolated a histone sulphate from calf thymus (J. Infect. Dis. *80:* 145, 1947). It differs in some respects from the histones previously reported. The arginine is lower and there is probably some tryptophane. Also, it has one of the highest lysine contents ever reported for any protein. This histone inhibited the aerobic respiration of the anthrax bacillus. Its action was suppressed by desoxyribonucleic acid but not by ribonucleic acid.

PETERMANN: We have prepared the histone nucleate of beef spleen by a modification of Dr. Mirsky's method in which desoxyribonuclease activity is inhibited by the use of sodium citrate. These histone nucleate solutions retain their high viscosity and flow birefringence when dialyzed against 0.001 M citrate. In the preparative ultracentrifuge, however, the histone and nucleic acid now sediment together as if closely bound. Nucleohistone extracted from washed spleen homogenates with 0.001 M citrate gives solutions which are highly viscous but do not show flow birefringence. These solutions are unstable, and their viscosity drops rapidly.

KAUFMANN: The report by Dr. Mirsky of the presence of ribose nucleic acid in the chromosome is of considerable interest to the cytologist, especially to those of us who have studied the chromosome-nucleolus relationship. Since it has long been established that the nucleolus contains ribose nucleic acid, the question has arisen whether the interchanges that occur between nucleolus and chromosome in the cycle of mitosis involve merely transportation of materials or chemical transformation. Earlier studies by Brachet and Schultz had indeed suggested that the chromosomes contain ribose nucleic acid; but conclusions based on the use of crystalline ribonuclease were open to the question whether such preparations were free of proteolytic activity. In an approach to the solution of this problem, Dr. McDonald has succeeded in preparing crystalline ribonuclease free of detectable proteolytic activity, as she announced earlier in this symposium. By using this ribonuclease under suitable experimental conditions a constituent has been removed from the chromosome so that its capacity for staining with basic dyes differs markedly from that of control preparations not treated with the enzyme. This finding also indicates that the chromosomes contain ribose nucleic acid, and we are now studying its distribution during the mitotic cycle.

MCDONALD: I have been puzzled by the fact that Dr. Mirsky, using apparently identical procedures, has prepared materials containing a) only nucleic acid and histones, and b) nucleic acids, histones and tryptophane-containing proteins. Would he tell us the technical reasons for not finding the tryptophane-containing proteins in his first preparations, thus preventing others from being misled by the same or similar difficulties?

MIRSKY: In our first work the tryptophane-containing protein was in large part removed by repeated precipitation, solution and centrifugation. In the work described in the paper that has just been presented, the tryptophane-containing protein fraction was clearly identified just because the procedures followed were quite different.

NUCLEOPROTEIN DETERMINATION IN CYTOLOGICAL PREPARATIONS

ARTHUR W. POLLISTER AND HANS RIS

Introduction

In an earlier paper in this symposium Mirsky has described how interphase chromosomes of many organs can be isolated in quantities sufficient for analysis by macrochemical methods; and he has summarized the results of study of the nucleic acids and proteins that make up the bulk of the constituents of these chromosomes. These analyses have shown clearly that there is a uniform pattern of chromosome composition, for the same four fractions are found in every case, namely: an abundance of desoxypentose nucleic acid, along with some pentose nucleic acid, and two clearly separable protein fractions, histone and residual protein.

Among the organs or tissues from which chromosomes have been isolated and analysed there are several which consist mainly or wholly of a single type of tissue cell, such as sperm suspensions, the red blood cells of lower vertebrates, thymus glands (chiefly lymphocytes), and liver (a highly epithelial organ, with very little connective tissue). It is most interesting that although all of these chromosomes from diverse cell types conform to the general nucleoprotein composition just described, there are nevertheless striking differences in proportions of the nucleic acids and proteins. A logical extension of this line of investigation would be to compare with these chromosomes others from different cell types and, most intriguing of all, to follow the nucleoprotein composition of chromosomes throughout functional cell cycles, such as mitosis and meiosis, secretory synthesis, tissue differentiation, etc. For such studies it seemed highly desirable to develop methods that would make it possible to determine the amounts of the nucleic acid and protein fractions in individual cells—an analysis, with the microscope, of a nucleus or chromosome *in situ*. This sort of attack is of course primarily cytological; and, being cytologists, the present authors, working in close contact with Mirsky's development of the methods of gross analysis, have concentrated attention on applying these same methods of nucleoprotein assay to cytological sections and smears. In this paper are described the techniques for quantitative analyses on individual nuclei, chromosomes, and nucleoli for the same four fractions that have been earlier described by Mirsky, and using essentially similar methods.

The properties of the nucleic acids and proteins that form the basis of the cytological techniques are all either those that are widely known, or those which have just been described for this group. The nucleic acid analyses depend upon such properties as the specific ultraviolet absorption of the heterocyclic bases, the apparent specificity of the dye pyronin for pentose nucleic acid, and the solubility of both nucleic acids in hot trichloracetic acid (Schneider, 1945). In the protein analyses the Millon reaction is used quantitatively; and the two fractions are separated by taking advantage of the unique property of histone of being soluble in a sulphuric acid-mercuric sulphate reagent.

Photometric Analysis with the Microscope

Of all ordinary quantitative methods that of photometric analysis is obviously most readily adaptable to high power microscopy, since it involves transmitted light. Many microchemical methods depend upon visual use of the microscope, and, indeed, the analytical methods described below involve only very simple adaptations of the microscope to make it an objective light-measuring instrument. A typical photometric chemical analysis is an application of the Beer-Lambert laws of absorption which, in a broad sense, say that the amount of light energy absorbed is a function of the number of molecules of specifically absorbing material in the light path. This number of molecules can be changed, of course, either by varying the concentration (c) or the thickness (d) of the absorbing layer; these two values are then the variables while the specific absorption of the substance is a constant (k); and under any given set of conditions the light loss by absorption is proportional to the product, kcd. In practice spectrophotometric analysis never deals with absolute amounts of energy, but with relative values. For example, the light which is transmitted by a specifically absorbing substance in solution (the *test*) is expressed as a decimal fraction (or alternatively as a percentage) of that transmitted by the solvent alone (the *blank*), the latter being taken as a transmission (T) value of 1.0 (or 100%). For calculations it is most convenient to have the value that varies directly with concentration and thickness, or the Extinction (E). Extinction is the logarithm of the reciprocal of the transmission fraction. If, for example, T is 0.1, then E is log 1.0/0.1, or log 10, or 1.0. A simple form of the Beer-Lambert law which expresses the relationship of extinction to concentration and thickness of $E = kcd$. (For detailed discussions see Brode, 1943, Gibb, 1942, or Drabkin, 1944.)

Since a photometric analysis is accurate only to the extent that it actually measures light loss due

to a specifically absorbing substance, the selection of a blank, to give the value $T = 1.0$ is very important. This must be set up so that the amount of non-specific light loss (such as that due to light scattering, internal reflections, surface reflection, etc.) is exactly the same for blank and test and thus effectively cancelled out. In practice, the two samples are in carefully matched absorption cells (cuvettes), and the blank solution contains all the constituents of the test except the specifically absorbing substance that is the purpose of the analysis; and, of course, the greater light loss from the beam of light passing through the test cell is then entirely due to the specific absorption.

Under conditions where Beer's law holds, if one is given the extinction value of a solution of known concentration and thickness (the *standard*) the concentration of an unknown is readily computed from the extinction. If the thickness of the unknown and standard are identical (as in a spectrophotometer) the calculation is made by substituting in the following equation:

$$C = \frac{E_2}{E_1} \cdot C_1$$

where C_1 and C_2 are concentrations of standard and unknown, and E_1 and E_2 are the standard and unknown extinction values. If the unknown differs in thickness from the standard two new terms (D_1 and D_2 for thickness of standard and unknown) must be introduced, and the equation becomes:

$$C_2 = \frac{D_1E_2}{D_2E_1} \cdot C_1$$

It is of course the second equation which applies in calculations from extinction data obtained on a cystological preparation (see below, p. 153) since the standard is measured in millimeters while the thickness of the section or smear is at most a few microns.

In attempting to approach exact methods for quantitative analysis of chromosomes in individual cells of a cytological preparation, the question of a proper blank seems especially urgent. The non-specific light loss in a structurally complex body like a chromosome must be expected to be considerable, especially at the shorter wavelengths; and the light loss, furthermore, must vary considerably with different technical treatment of the preparation. In fact, we have detected examples where the non-specific light loss accounted for nearly half of the total extinction value for a cellular structure. In each of the techniques described below there is one point in the procedure at which the blank is measured to give the extinction value of the non-specific light loss.

The time at which a blank is measured depends upon the technique as a whole. For example, if one wishes to determine the absorption due to a stain, the obvious blank is the cytological structure before it was stained (*e.g.*, pyronin staining for ribonucleic acid determination). In analysis for a naturally occurring substance with specific absorption, such as nucleic acids, the blank is the structure measured after removal of the nucleic acid. Where a test causes the appearance of a new specific absorption in a naturally occurring substance, as the Millon reaction does with protein, the blank is measured just before the development of the new color by a slight change in the reagent.

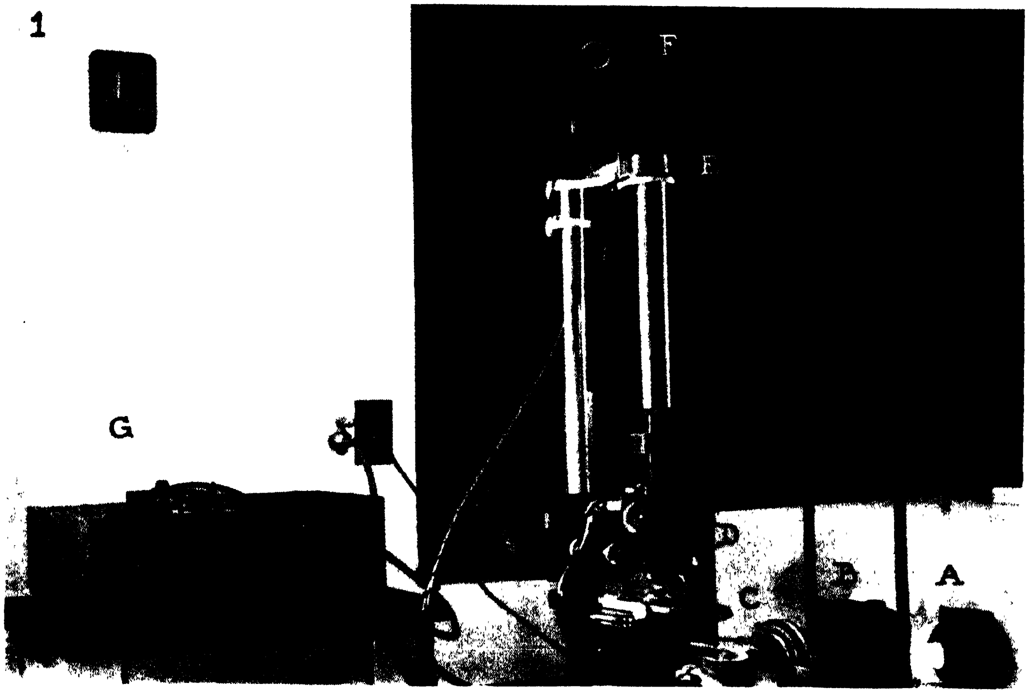
Apparatus and Cytological Technique

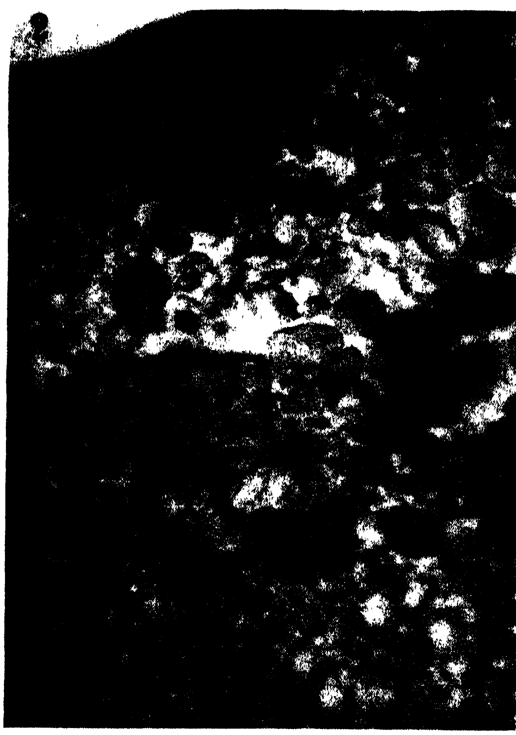
The apparatus (Fig. 1) is not complicated; and all parts are either available commercially or may be easily constructed with common tools. It was designed by a cytologist and is for use by cytologists. Experience in the Columbia University laboratory has shown that in less than one hour a trained cytologist can master the entire technique of photometric analysis with this apparatus.

Table 1. Spectral Regions for Photometric Analysis

Source	Peak	Filters	Purpose
Tungsten Ribbon Filament	480 mμ	1 cm. 0.28 M $CuSO_4$ plus Wratten #75 (Drabkin, 1944) or Farrand Interference filter	tyrosine-mercurial
AH4 G.E. Mercury Arc	546 mμ	Wratten #66 or #74	nucleal fuchsin (Feulgen) or pyronin
AH4 G.E. Mercury Arc	365 mμ	Corning 5840, 2.4 mm.	tyrosine and tryptophane mercurials
AH4 G.E. Mercury Arc	630 mμ	Corning	methyl green
G.E. 4 watt germicidal	2537 A	1.75 M $NiSO_4$ plus 0.5 M $CoSO_4$ in 5.0 cm. Quartz cell (Bäckstrom 1940)	purines and pyrimidines

The microscope is always used with Kőhler illumination. If one intends to compute absolute amounts of material with reference to a standard, it is more accurate to make the measurements with a narrow condenser aperture (*e.g.*, 3.0 mm diam.) in order to approximate parallel light; for most standards spectrophotometers measure the transmission of parallel light. The parallelism of the illumination may be roughly checked by focussing the condenser up and down through some distance with





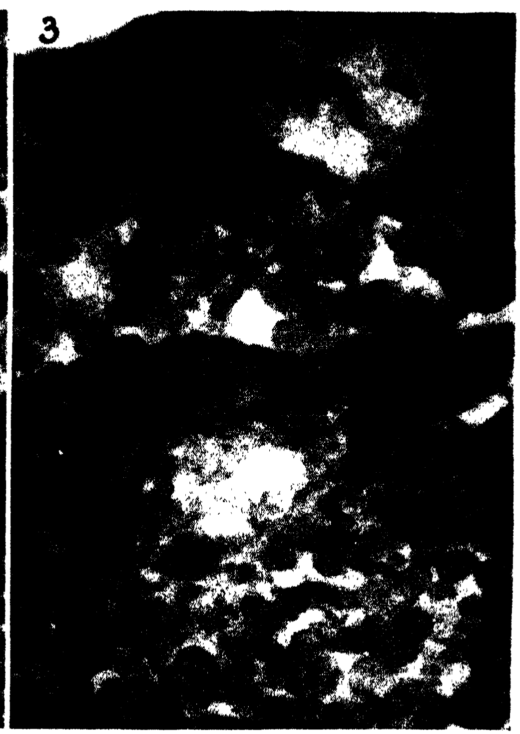


PLATE I

FIG. 1. The quartz microscope for absorption measurements at 2537 A. *A*. Light source (cover removed); *B*. Quartz lens; *C*. Filter cell; *D*. Microscope with quartz optics; *E*. Diaphragm in image plane; *F*. Photo-tube; *G*. Photovolt amplifier and galvanometer.

FIG. 2. Section of salamander blastula, fixed in Susa, unstained, photographed at 2537 A.

FIG. 3. Same cells as in Fig. 2, after treatment with 0.3 M trichloracetic acid, photographed at 2537 A. See text p. 149.

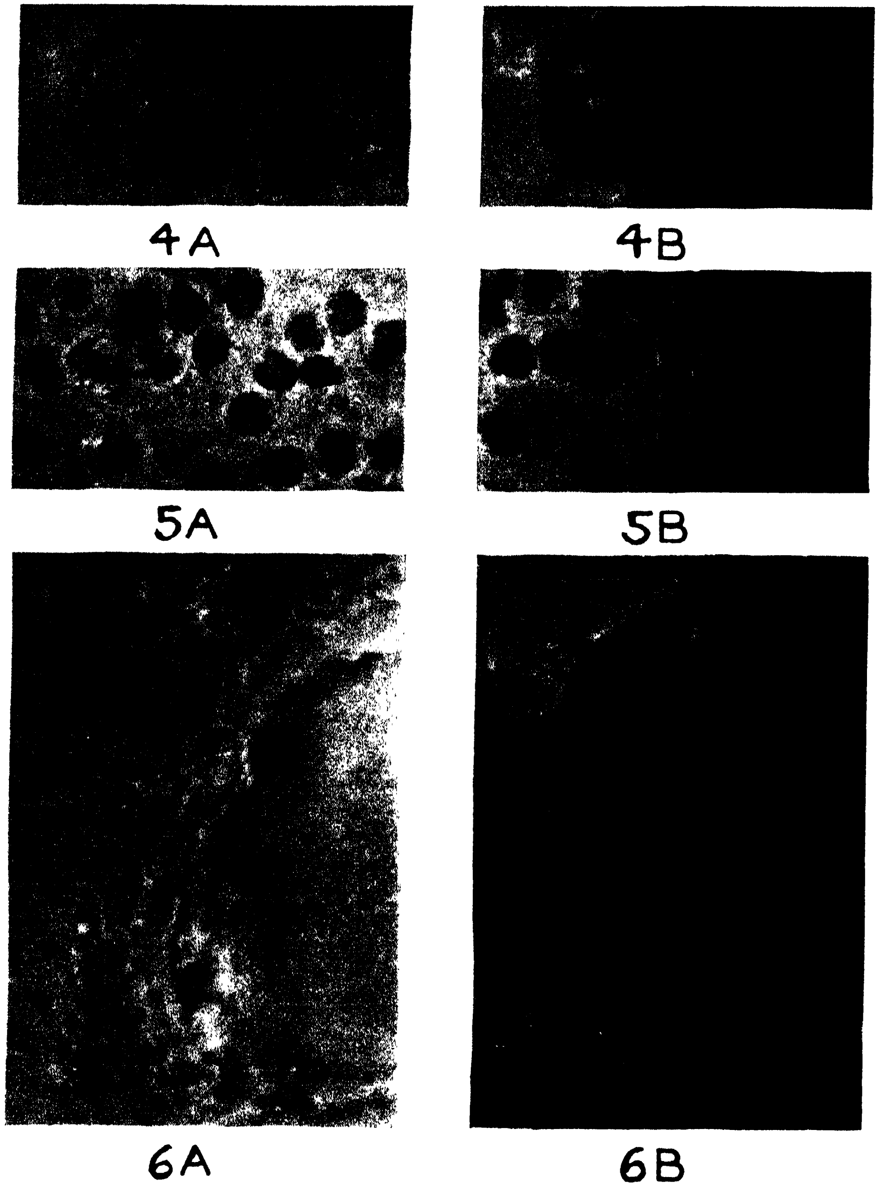


PLATE II

FIGS. 4–6. Blank (*A*, on left) and Millon test (*B*, on right) photomicrographs, showing by the contrast between the two members of each pair the difference in absorption which is the basis for quantitative estimation of tyrosine. All photographs made at wavelength 365 mm, at identical exposures, and developed, fixed, and printed simultaneously.

FIGS. 4*A* and 4*B*. Pollen mother cell of *Zea mays* at pachytene, with large nucleolus. Susa fixation, thickness approximately 3 microns, magnification 1400 times. From density measurements of these two negatives the extinction due to Millon mercurials in the nucleolus was determined as 0.134.

FIGS. 5*A* and 5*B*. Primary spermatocytes of the Atlantic salmon, showing protein tests in the metaphase chromosomes. Bouin fixation, magnification 1170 times.

FIGS. 6*A* and 6*B*. Cross section of thyroid gland, showing strong Millon test in colloid. Susa fixation; thickness 5 microns; magnification 1400 times; extinction due to tyrosine and tryptophane mercurials was 0.171.

the photoreceiver in place, and noting the galvanometer reading. If there is little change the light beam is only slightly convergent.

The lenses and light sources to be used depend upon the spectral region to be measured. Sources for the methods to be described below are listed in Table I. A suitable voltage stabilizer should be used with any of these lamps. The measurement of absorption of 2537 A radiation must be made with quartz optics. With the germicidal lamps as source the image is first focussed by visible light, and then refocussed for 2537 A by turning the fine adjustment a certain number of turns, as predetermined with a fluorescent screen in the manner described by Lavin and Pollister, 1942. If an apochromatic oil immersion objective is used the 365 mμ image is in focus at the same level as the visible image (*e.g.*, that with the green mercury line at 546 mm). This has not proved true of any achromatic lenses we have examined.

The light transmitted by cytological structures is measured by a photovolt Photometer, Model 512 (Fig. 1), consisting of a phototube in a light tight case (the "search unit," F), an amplifier circuit, and a galvanometer G. The manufacturer's phototube is barely sensitive enough to measure large areas, such as whole nuclei. At our suggestion Photovolt Corporation has adapted an electron multiplier tube to the apparatus. This has enormously increased the sensitivity of the instrument, and with this improvement it seems likely to be adequate for any demands that may be made upon it. The search unit is mounted in the vertical microscope axis approximately 30 centimeters above the eyepiece in such a way as to be movable horizontally by micrometer screws for centering on a selected area of the microscopic image. In practice the image is picked up and centered with a 6× focussing magnifier, and the diaphragm which surrounds the aperture to the phototube, is closed to circumscribe an area for the transmission measurement. The magnifier is then removed and the search unit is lowered into position for the reading. Two readings are the basis of each transmission determination: first that through the specimen, and second, immediately afterward, a reading through an empty area of the slide, which has been brought rapidly into the field by movement of the mechanical stage. The *transmission of the specimen* is the first reading divided by the second. Test and blank slides are each measured in this way; and the *final transmission value* indicative of the amount of specifically absorbing material is the transmission of the test divided by that of the blank. From this third transmission the extinction is computed.

Considerable test tube and cytological experimentation has been necessary to discover the proper cytological technique for preservation of material in which the nuclei are to be analyzed for nucleoproteins. A major problem is that of preventing the loss of the acid-soluble histone. Two fluids which hold practically all of the histone within the nuclei are Susa's formol-sublimate-trichloracetic acid mixture (Romeis, 1932) and Carnoy's acetic alcohol (25 parts acetic acid to 75 parts of absolute alcohol). But it is not enough to hold all the chemical constituents of the nucleus *in situ* by fixation. If complete analysis is to be made the fixation must allow later complete removal of the nucleic acids and histone. The Carnoy formula alone is satisfactory in the second respect. After Susa's fixation a large part of the histone is removable, but as much as one-fourth may completely resist the sulphuric acid-mercury reagent.

Determination of Nucleic Acids

a. Total nucleic acid

Nucleic acids have a characteristic absorption curve in the ultraviolet with a maximum at 2600 A, and a very high specific extinction. This property is the basis of a most useful cytochemical method for the qualitative and quantitative determination of nucleic acids. Caspersson (1936) has demonstrated that under certain conditions and in connection with a sensitive photometer, the microscope can be used as a microspectrophotometer. Using the quartz lenses developed by Kőhler and appropriate light sources in the ultraviolet, Caspersson was able to determine the absorption curves of cellular structures. He thus established that the absorption of chromosomes at 2600 A is due to nucleic acid. Now the ultraviolet absorption curve not only reveals the presence of nucleic acid, but the absorption at 2600 A is also a measure of the quantity of nucleic acid present. This quantitative determination is however complicated by the fact that light in passing through a cell structure is not only absorbed, but also reflected and refracted. We have to subtract this non-specific "structural light loss" from the absorption measured in order to determine the specific absorption due to nucleic acid. This can be done in a simple way. Schneider (1945) has shown that nucleic acids are removed from nucleoproteins by heating in 0.3 M trichloracetic acid at 90°C for about 15 minutes. This same procedure can be used on a cytological preparation, either smears or sections, without destroying the cell structure. The procedure then is in essence the following: The extinction at 2537 A of the structure to be analyzed is determined. Then the nucleic acid is removed in hot trichloracetic acid and the extinction at 2537 A of the same structure is measured again. The difference corresponds to the extinction due to nucleic acid and from it the quantity of nucleic acid can be calculated. Figs. 2 and 3 illustrate the result of this treatment. Fig. 2 is an unstained section through a blastula of a salamander (Ambystoma),

photographed at 2537 A. The lower cell shows a metaphase spindle with chromosomes, the upper one a resting nucleus. Fig. 3 represents the same cells after treatment with trichloracetic acid. Chromosomes, spindle fibers, and asters have practically vanished, only hazy shadows are left. The density of the other structures remains unchanged. Chromosomes, spindle fibers, and asters therefore contain substantial amounts of nucleic acids. These pictures show clearly that it is impossible to determine simply from the densities of a photograph at 2537 A where nucleic acids are located in the cell. The cell structure without nucleic acid is of course not a perfect blank, since nucleic acid contributes to the structural light loss. But in practice this difference can be neglected. The method of accounting for non-specific light loss in this way makes it unnecessary to record an absorption curve for quantitative measurements, once it is known what substance causes the absorption. This simplifies considerably the problem of a light source (see Table I).

b. Desoxyribonucleic acid

The extinction at 2600 A measures both ribonucleic acid and desoxyribonucleic acid. Since the Feulgen reaction is specific for the desoxyribonucleic acid it can be used to measure this substance even in mixtures of nucleic acids. Due to the nature of the reaction absolute quantitative measurements are not possible; but relative measurements on the same slide are feasible. The colored product of the desoxyribose has an absorption maximum at 540 mm. With the ordinary lenses and the Photovolt photometer the extinction of various structures at this wave length can be determined and give a measure of the relative amounts of desoxyribonucleic acid which they contain.

c. Ratio of ribonucleic acid to desoxyribonucleic acid

The analysis of isolated chromosomes has shown that they contain both nucleic acids, but that the ratio varies in different tissues (Mirsky, this volume). This finding made it very desirable to have a cytochemical method for determining the relative amounts of these nucleic acids. The small percentage of ribonucleic acid present in most nuclei and the impossibility of removing completely one or the other acid makes the usual methods of determination impracticable. But there are two remarkable basic dyes, pyronin and methyl green, which, as Brachet has demonstrated, have an astonishing ability to distinguish between the two nucleic acids. The reason for this is so far unknown, but we have been able to confirm the fact by staining isolated chromosomes and nuclei of known composition. The two dyes have completely separate absorption maxima, pyronin at 550 mμ and methyl green at 630 mμ; and the ratio of extinction at these two wavelengths can be used to measure the relative amounts of the two nucleic acids, as shown by the following experiments. The amounts of the two nucleic acids were estimated in nuclei of isolated calf thymus and liver. The ratio of ribonucleic acid to desoxyribonucleic acid in the liver nuclei was found to be 2.4 times that of thymus nuclei. Thymus and liver nuclei were then stained with the pyronin-methyl green mixture and the ratio of the extinction at 540 mμ to the extinction at 630 mμ determined. This gives the relative amounts of pyronin and methyl green combined with liver and thymus nuclei. The ratio pyronin to methyl green in liver nuclei was found to be 2.6 times that of thymus nuclei, which is in good agreement with the value found with chemical methods. The ratio of pyronin to methyl green as determined by absorption measurements can therefore be used as a measure for the relative amounts of the two nucleic acids present.

Protein Determination

As is well known, proteins which contain the aromatic amino acids, tyrosine and tryptophane, show a natural specific absorption in the ultraviolet spectrum, with the peak near wavelength 2800 A. In a cytological structure which has little or no nucleic acid it is possible to take advantage of this property as a means of securing qualitative evidence of the presence of protein (as for example, Gersh and Caspersson, 1940, have done with thyroid colloid).

In the presence of nucleic acid, however, the detection and estimation of protein by its natural 2800 A absorption encounters the major obstacle of interference from the much stronger absorption of the purine and pyrimidine bases. Although 2800 A is not the absorption peak of the nucleotide bases, nevertheless at this wavelength the specific extinction of a nucleotide is at least equal to that of tyrosine or tryptophane. Moreover in a nucleic acid every nucleotide residue has this specific absorption, while in a typical protein not more than a small percentage of the amino acid residues are the specifically absorbing tyrosine or tryptophane. As a consequence, at roughly equivalent concentrations the 2800 A extinction value of nucleic acid may be over fifty times that of a typical protein (Caspersson, 1936). In a chromosome the nucleic acid concentration usually approaches that of the total protein; and the result is that at 2800 A the protein absorption is negligible in comparison with that of the nucleic acid. This is strikingly shown by plotting on the same scale the curves of thymus chromosin (the nucleoprotein that makes up over 80% of the thymus chromosome) and of thymus nucleic acid with an equal phosphorus concentration (Mirsky and Pollister, 1946). In the region of specific nucleic acid absorption (2500 A to 3000 A) the two curves appear

identical, showing that the tyrosine and tryptophane absorption have no measurable effect upon the extinction values.

Caspersson and his coworkers (for references, summary, and conclusions, see Caspersson, 1941) have published what they call "typical absorption curves" of metaphase chromosomes, chromocenters of the nuclei of Drosophila salivary glands, and nucleoli. In these they believe it possible to detect the protein and to assay it quantitatively, because the protein peak has shifted to the region of 2900 A, and has thus partially "escaped" from the dominance of nucleic acid. Caspersson believed this longer wavelength absorption to be characteristic of basic protein ("histone type"), a conclusion based upon some data of his own and on some speculations by Stenstrom and Reinhard, 1926, on the possible effect of basic amino acids upon the dissociation of the neighboring tyrosine groups. Spectrophotometric curves of many relatively pure histones recently examined (Mirsky and Pollister, 1943) have shown the peak to be in the usual protein region, and not shifted toward 2900 A; and until some constituent of chromosomes or nucleoli is found which has a 2900 A absorption the significance of these *typical* analysable curves found by the Caspersson School remains wholly obscure. It should be added, furthermore, that any quantitative interpretation of the data is open to the additional objection that there is no measured blank; and indeed, usually not even a calculated blank is included with the published curves. There is, moreover, usually no attempt to determine the thickness of the object measured; and the data are presented as milligrams per square micron of absorbing surface, not as actual concentrations or as total amounts in a cellular structure.

From the above it is obvious that the protein can be determined photometrically in chromosomes only if its specific absorption can be changed so as to be outside the 2500-3000 A range. Such a shift in the absorption spectrum of a protein containing tyrosine or tryptophane is easily accomplished by use of the Millon reaction. This is one of the very oldest of the spot tests for protein, and it depends upon the formation of nitroso-mercurial derivatives of tyrosine and tryptophane (hereafter referred to as *mercurials*). These derivatives have specific absorption in both the visible spectrum and the near-visible ultraviolet; and both reactions are used in accurate quantitative methods of photometric analysis in protein hydrolysates (Brand and Kassel, 1939). The Millon reaction gives a strong color in tissue sections (Figs. 3, 4, and 5) and it has frequently been used cytologically as a qualitative test for protein (Leitgeb, 1888, Serra, 1946, and Thomas, 1946).

A commonly used formula for Millon's reagent is chiefly a solution of sulphuric acid and mercuric sulphate. Histones are soluble in this reagent (Mirsky and Pollister, 1946); and therefore it is possible to measure an absorption curve of histone in which the tyrosine-mercurial has been formed and to compare the spectral properties of this derivative in peptide linkage with that of pure tyrosine-mercurial. The two curves are not greatly different in shape, and both have pronounced peaks at 3550 A and 4800 A. From this standard histone curve also come specific extinction values from which absolute amounts of histone and of protein may be computed from measured extinction values of unknowns.

The tryptophane-mercurial has an absorption beginning in the shortest visible part of the spectrum and steadily rising throughout that part of the ultraviolet spectrum which is measurable by the spectrophotometer. At the tyrosine-mercurial ultraviolet peak, 3550 A, tryptophane-mercurial has approximately the same specific extinction value as tyrosine-mercurial; but at the visible tyrosine-mercurial peak, 4800 A, the absorption of tryptophane-mercurial is practically negligible. In the analyses given below we are mainly concerned with tyrosine, but it is worth noting that if one makes determinations at both spectral regions 3550 A and 4800 A, the ratio between these two extinction values offers a basis for computing separately the amounts of tyrosine and tryptophane. All of the protein data in this paper are from measurements at wavelength 3650 A. The extinction values therefore are due to both tyrosine and tryptophane.

For cytological use it has been necessary to develop some modifications of the standard Millon reaction, first to allow for a blank and second to make the test without dissolving histone. Total protein is preserved throughout the test if a trichloracetic acid-divalent mercury reagent is substituted for the standard sulphuric acid mercury reagent. The test is always made in two steps, first treatment of the tissue with the acid mercury reagent but without nitrite, and second a longer treatment with nitrite. The blank is measured between these two steps; for it has been found that the strong acid reagent considerably changes the optical properties of cellular structures (as shown, for example, by a change in refractive index), while no further change of physical properties is detectable upon the addition of the slight amount of nitrite.

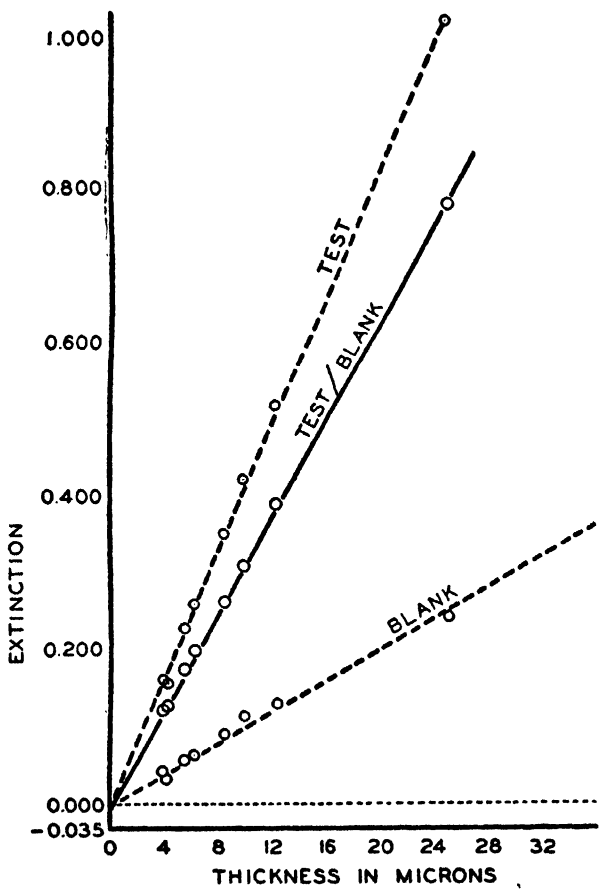
The two methods employing the Millon test, combined with the special solubilities of histone, provide the basis for determinations of the two nuclear fractions; histone and residual protein. Total protein is determined by using the trichloracetic Millon formula. Non-histone protein is determined by using the sulphuric acid-Millon following removal of all nucleic acid by hot trichloracetic acid. The difference between the extinction from these two measurements is the extinction due to histone.

In comparing different cells or cells in different

physiological states it is useful to determine the nucleic acid-protein ratio. A relative measure for this ratio is given by the ratio of the extinction at 2537 A to the extinction at 3640 A, after the trichloracetic-Millon. If the sulphuric acid Millon is used after removal of nucleic acid, the ratio of extinctions at these two wavelengths measures the proportions of nucleic acid to non-histone protein. From these two ratios the relative amounts of histone and non-histone proteins can be calculated.

Calibration

Some simple calibration experiments have been made to test the adequacy of the photometric ap-



Text Fig. 1. Results of measurements of Millon mercurials in thyroid colloid of the guinea-pig, cut at various thicknesses. Absorption of 365 mμ radiation from a mercury vapor lamp. Each extinction point is the mean of 30-50 follicles.

paratus and the validity of the cytological procedure as compared with gross analyses.

One experiment was designed to find out whether the test extended evenly throughout the section, and at the same time to show whether the photometry adequately measured extinction values over a considerable known range. Thyroid gland (Figs. 4A and 4B) was cut at different microtome settings, the actual thickness was carefully determined with the fine adjustment of the microscope, and extinction values for colloid protein-mercurial were determined for a large number (22-47) of tubules in each section. The extinction was found to vary in direct proportion to the thickness of the section. (Text Fig. 1), as would be expected from Lambert's law if one were measuring a series of thicknesses of a material of uniform concentration. This result also shows, of course, that the test must have been uniform throughout even the thickest sections.

In the development of cytochemical techniques it is of great advantage if they can be compared with measurements on the same object using conventional chemical procedures. We therefore determined, by the method described above, the nucleic acid content of single isolated calf thymus nuclei (citric acid method). It was found that one nucleus contained $1.1 \cdot 10^{-9}$ mg nucleic acid. The variation from nucleus to nucleus was within a few percent. Then the number of nuclei contained in one cc of the suspension was determined with a hemocytometer and was found to be $6 \cdot 10^{9}$. From the phosphorus content of 1 cc of these nuclei it was calculated that these $6 \cdot 10^{9}$ nuclei contain 6 mg nucleic acid. One nucleus therefore has $1.0 \cdot 10^{-9}$ mg nucleic acid, which agrees very well with the value determined cytochemically. The proportions of histone and nonhistone protein were also determined on isolated thymus and liver nuclei in the test tube.

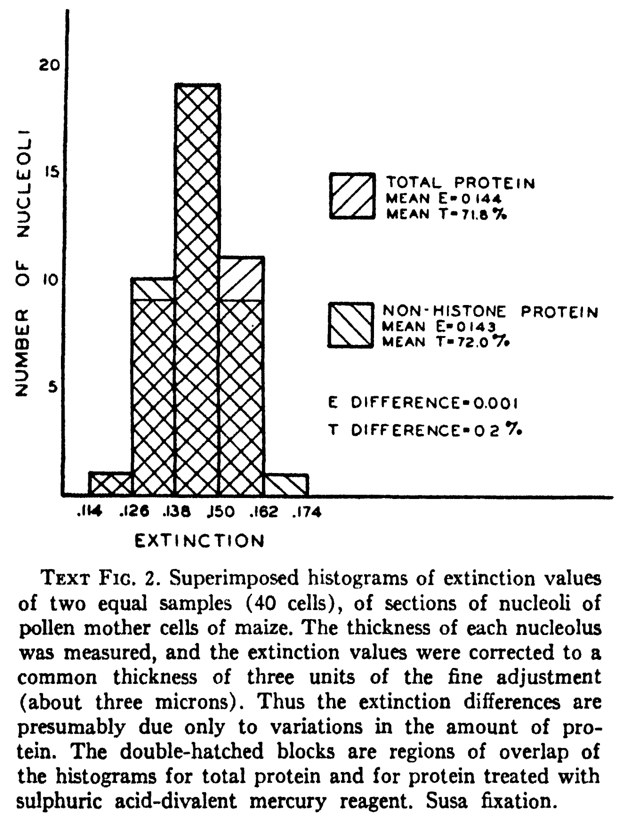
Tests were also made to determine whether the cytological method could accurately measure differences in concentration from known test-tube data. Available for this purpose were isolated nuclei from thymus and liver, on which gross analyses had shown the approximate proportions of histone and residual protein. The analyses of masses of thymus nuclei indicated that about 70% of the protein of the nucleus is removable by the standard Millon reagent; by comparison the mean of a microscopic analysis of a small number of individual nuclei showed that 68% of the protein had been removed. For liver nuclei, in which analytical results are much more variable, the respective gross and microscopic values were 40% and 49%. The two methods agree as well as perhaps might be expected in view of the fact that the microscopic values are from a few nuclei, while the gross analyses are averages of billions of nuclei, including those of more than one type of cell.

Protein Content of the Nucleolus

The nucleolus is not a permanent nuclear structure; it disappears in late prophase of each division, and is reformed during the next telophase by the activity of a special chromosomal region known as the nucleolar organizer. Because of this genetic re-

lation to the chromosome it is interesting to compare the protein content of the nucleolus with that of the chromosomes. Caspersson, 1941, by spectrophotometry with a quartz microscope, has attempted to determine the relative amounts of histone type of protein, and globulin type protein in the nucleolus, and has concluded that the nucleolus has a high histone content. This conclusion was based on a premise concerning the absorption spectrum of histone which has now been shown to be false (see p. 151). The classical histone is readily detached by sulphuric acid Millon reagent; and we should therefore be able to determine by the method described above whether this histone fraction is abundant in a nucleolus.

Dr. Marcus Rhoades brought to our attention the nucleolus of pollen mother cells of *Zea mays*



TEXT FIG. 2. Superimposed histograms of extinction values of two equal samples (40 cells), of sections of nucleoli of pollen mother cells of maize. The thickness of each nucleolus was measured, and the extinction values were corrected to a common thickness of three units of the fine adjustment (about three microns). Thus the extinction differences are presumably due only to variations in the amount of protein. The double-hatched blocks are regions of overlap of the histograms for total protein and for protein treated with sulphuric acid-divalent mercury reagent. Susa fixation.

as an unusually favorable object for cytochemical study by reason of its large size. The material was furnished by Dr. Rhoades, and he assisted us in identifying some of the stages. By smear technique, flowers with anthers containing pachytene stages were identified; and then a large number of whole anthers were dissected out, and fixed for one hour in Susa's fluid. Paraffin sections cut at 3μ were used for absorption measurements.

The nucleolus at pachytene is a sphere approximately 6μ in diameter (Figs. 4A and 4B). In many cases it is quite homogeneous, but some nucleoli are vacuolated and, still more rarely, they contain small dense bodies, visibly quite different from the rest of the nucleolus. Since all three of these types showed extinction values with about the same distribution, they have been combined indiscriminately in the results. In 3μ sections of nucleoli one finds various segments of a sphere; and the type of segment is easily determined by focussing on the top and bottom surfaces. For extinction measurements we selected nucleoli that had been cut so that the smaller cut surface was at least 3μ in diameter. The diaphragm of the phototube mount circumscribed an area of the microscopic field which was approximately 3μ in diameter; and thus each measurement recorded the absorption of a cylinder the height of which could be determined by focussing with the fine adjustment. It was necessary to measure this dimension for each nucleolus, for even adjacent nucleoli within one pollen cavity may differ considerably in thickness. The extinction values should of course be directly proportional to the thickness; and therefore for ready comparison of amounts of tyrosine-derivative, each extinction value was corrected to a thickness of 3μ. Variations in these final corrected extinction values are thus presumably due entirely to variations in concentration of tyrosine and tryptophane. Extinction values of tyrosine in total protein were determined for 40 nuclei by the trichloracetic Millon reagent; and also determined for the same number after treatment with diluted standard Millon reagent, the procedure known to remove nearly the whole of any histone that might have been present.

The histograms of the extinction values of these two equal nucleolar samples are shown superimposed in Text Fig. 2. The double-hatched blocks are regions of overlap of the total protein tyrosine and of protein treated so that any histone would have been removed. These cross-hatched blocks include all but 4 of the 80 nucleoli measured. Hence it appears that there is practically no difference between total protein tyrosine and tyrosine in protein from which any histone present would have been removed. This is further shown by the practical identity (0.144 and 0.143) of the mean extinction values of the samples by the two methods. The transmission difference of 0.2% is one-fifth of the minimum transmission difference detectable with the phototube apparatus. It may, therefore, be concluded that the nucleolus of pollen mother cells of *Zea mays* at pachytene contains no more than a negligible quantity of protein attached as is the histone of chromosomes.

While these results do not offer support for the speculations of Caspersson concerning the histone-synthesizing role of the nucleolus, we feel, on the other hand, that they do not necessarily make such a theory untenable. The nucleolus in this meiotic

cell at prophase is a transient body, one might almost say moribund, for it is soon to disappear as the prophase changes leading to the first division proceed. It may be that this imminence of dissolution accounts for the variation of tyrosine content found from our measurements. At any rate, such a nucleolus cannot be compared in too strict a sense with a nucleolus in an active metabolic nucleus; and it is quite possible that the latter may have a very different chemical composition.

Using as a standard the extinction values of histone-protein solutions as determined in a spectrophotometer (1.0 mg/cc, path. 1.0 cm, E_{3650} is 1.1), it is interesting to compute the total tyrosine content of the nucleolus (see p. 148). Calculated as a sphere of radius 3 micra, the volume of the nucleolus is 1.16×10^{-10} cm^3. The concentration of protein is 430 mg/cm^3, calculated from the mean extinction value of 0.143 for the 3 micra (0.003 cm) section. The amount of protein in the whole nucleolus is 5.0×10^{-11} gram or 5.0×10^{-5} micrograms. It appears that the nucleolus may well be a "solid granule" in a physical sense as well as in its cytological appearance.

Summary

The photometric analysis for total nucleic acids by its natural absorption at 2537 A, using as a blank the same slide after removal of nucleic acids by hot trichloracetic acid, is described. Ratios between the two nucleic acids may be determined by staining with a pyronin-methyl green mixture.

The method of protein determination in cytological preparations by quantitative use of the Millon reaction is described.

The methods have been calibrated by measurements upon sections of colloid of thyroid gland, and by comparing measurements upon isolated nuclei with values for the same sample determined in a test tube.

In a study of the proteins of the nucleolus it was found that it contains little, if any, protein bound in the same manner as is the histone of chromosomes.

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Discussion

Hydén: In connection with what has been said about staining properties of cells and their content of nucleic acid, I should like to make some remarks of a general character.

In most cases there is a good correlation between the nucleic acid content of the cells and their capacity for binding basic dye groups, when stained according to ordinary histological procedures. But working with microspectroscopical methods it is a general finding that one has to be very cautious about drawing such conclusions in all cases.

A most striking example can be taken from the nerve cells in the cochlear ganglion. These cells contain, under acoustically quiet conditions, large amounts of nucleic acids in their cytoplasm. On acoustic stimulation they will be almost depleted of their cytoplasmic nucleic acids. Nevertheless, by slight changes in the staining procedures employed it is possible to obtain the same basophilia in both cases (Hamberger and Hydén, 1945).

Another example is given by Dr. Thorell (1945) in cytochemical investigations on plasma cells. The perinuclear parts of the cytoplasm of plasma cells take up no pyronin but microspectroscopical studies show decisively that they contain nucleic acids in large amounts.

Regarding photographs in the ultraviolet at 2570 A, I wish also to emphasize the following fact. It is impossible by estimating the density on the photographic plate with the naked eye to decide whether a moderate absorption is due to nucleic acids in small amounts or to the amino acids, concerned in great amounts. Any sort of quantitative data can only be calculated from a complete absorption spectrum taken between 2400 and 3200 A.

Ris: We agree with Dr. Hydén that one must be

cautious in interpreting results of staining in terms of chemical composition. Certainly there are variables which have to be controlled, like fixation, procedure of staining and differentiation. But the staining with basic dyes especially is now quite well understood chemically. Furthermore we were able to show that pyronin for instance can be used quantitatively for relative estimations of ribonucleic acid and gives reproducible results. One should therefore not be afraid to use basic dyes—or perhaps even acid dyes as Dr. Hydén has done—as cytochemical tools, if they are used under known conditions and the results are checked with other methods.

With regard to photographs at 2600 A, we are in complete agreement with Dr. Hydén and have emphasized this in our paper. But we must take issue with his statement that any sort of quantitative data can *only* be obtained from a complete absorption curve. In chromosomes, with which we are dealing mainly, the nucleic acid concentration is such that its absorption from 2400 A-3200 A overshadows the absorption of the proteins. This makes it impossible to obtain more than very rough estimates of the nucleic acid-protein ratios from such absorption curves. For this reason we developed the techniques described in our paper. We know that the structural light loss after removal of nucleic acid is not the same as before. Therefore we compared the results of cytochemical determinations with estimations of nucleic acid using ordinary chemical techniques on the same material. Since the results agreed quite well the error inherent in the technique cannot be large. Unfortunately we cannot compare the accuracy of our method with the nucleic acid estimations from absorption curves because they do not seem to have been checked against measurements with conventional methods on the same objects.

BRACHET: As regards the sensitivity of the trained human eye, there is no doubt that there are cases where gradients can be picked up much more easily with the eye than with other methods. In the particular case of Amphibian eggs, quantitative measurements of the U.V. absorption would be especially difficult because of the great heterogeneity of the cells, which contain yolk, pigment, etc. However, such gradients are very obvious on mere examination of stained preparations.

Concerning Hydén's findings in virus diseases, it is worth mentioning that I obtained, in collaboration with Gratia and Jeener, results essentially similar in the case of the silkworm jaundice. The first reaction of the infected cells is an increase in cytoplasmic basophilia, accompanied by a multiplication in the nucleoli and an increase in cell volume; later on, when the virus forms DNA containing polyesters in the nuclei, cytoplasmic basophilia decreases again.

STOWELL: Dr. Pollister and Dr. Ris have admirably demonstrated some important new possibilities of quantitative photometric histochemistry. With such methods I feel, as they do, that if we are aware of the sources of error and do not overemphasize the accuracy of the results obtained, we can obtain much valuable information on many problems.

There is one possible source of error, in addition to those mentioned, which may occur with such methods. It is difficult to compare the absorption of pigment in inhomogeneous cellular constituents with tests for Lambert's-Beer's law in solutions of homogeneous material. If the stained cellular constituents are large, irregularly dispersed, and have a high absorption such as over 50%, it is possible that they may overlie each other in such a way as to give an erroneous absorption. For example, the summation of the absorption from three large, densely absorbing masses of chromatin would be different depending upon whether the light passed successively through each or whether they were not in the same plane so that the light pathway only passed through each one separately. I believe that in comparative measurements, averaging the results on many cells, such errors are not large or important. Do you have any data on this possible source of error?

In connection with the interesting work on pyronin and methyl green, can you tell us how well in your experience these dyes obey the Lambert's-Beer's law?

RIS: Unequal distribution of absorbing substances can certainly cause errors in absorption measurements, as Dr. Stowell points out, and this has to be considered in making such measurements. In the techniques we use however, the absorption of single cell components is low and the distribution not far from even so that the error is not serious.

Methyl green follows Beer's law exactly and pyronin with aberrations so small, that they can have no effect on measurements as they were described.

FANO: Speakers on this Symposium, as well as other workers (notably Caspersson), have devoted considerable attention to the manifold systematic factors affecting the quantitative significance of histochemical analysis by spectromicrophotometry. I should welcome, however, at this time some positive statement on the following general questions: (a) how thorough is the exploration of such systematic factors believed to have been; (b) whether the overall error involved in the analysis is believed to be of the order of 10% (in which case the analysis could be called quantitative) or whether it might amount to something like a factor of 2. A restatement of the situation on this subject seems particularly desirable now when two different techniques have come into use.

Substances like nucleic acids may well be concentrated into *submicroscopic* units even though their investigation, *e.g.* by staining, may show an apparently uniform distribution throughout the volume of coiled chromosomes. Such rapid variations of concentration over distances comparable to the wave length of light may be a serious barrier to carrying out any truly quantitative analysis. To appreciate the situation the general features of the problem of spectromicrophotometric analysis may be outlined as follows:

The quantity which is measured by microspectrophotometry may be indicated as $M = \iint_S I(\lambda,f,x,y)\,dx\,dy$, i.e. the sum of the intensity of light of wave length λ at all points (xy) within an area S of the image formed by a microscope whose focal adjustment is indicated by f. M is determined by the characteristics of the light source and by the concentrations $c_1(X,Y,Z)$, $c_2(XYZ)$ . . . of all different atomic groups, having characteristic optical spectra, at all points (XYZ) of the object under the microscope. It is clear in general that $I(\lambda_i, f_i, x_i, y_i)$ bears a particularly close relationship to $c_i(X_i, Y_i, Z_i)$ when the i-th atomic group has maximum absorption at the wave length λ_i and f_i is such that the image of the point $(X_iY_iZ_i)$ is formed at (x_iy_i). It seems not so clear, however, what detailed inference on c_i can be made in the cytological applications on the basis of measurements of M.

From Caspersson's work I understand that, if c_i has a constant value γ throughout a sphere about 1 μ in diameter and zero outside that sphere, a measurement of M for the characteristically absorbed wave length λ_i and over an area S covering the central portion of the image of the sphere will give an accurate estimate of $c_i = \gamma$. However, the appearance on the image of a dark region such as would be produced by a nearly spherical blob of absorbing material may not be a safe index of the existence of a type of distribution of c_i for which Caspersson's conclusions hold with sufficient approximation. Also, it has not been clear from the discussion in this Symposium how much information can be obtained by means of comparative measurements of M at widely different values of λ.

Dr. Pollister's method is based on the comparison of the values of M measured before and after a chemical treatment intended to remove the concentration $c_i(XYZ)$ of the atomic groupings responsible for a characteristic absorption at λ_i without affecting the distribution of all other substances. This method has undoubtedly considerable advantages but it is not clear to me how important is the effect of variations of c_i throughout the volume of object under consideration. It is not clear to me either how comparable is his procedure for the preparation of a "blank" in cytological specimens to the corresponding procedure in the usual photometric determination of dyes.

POLLISTER: It is doubtless true, as Dr. Fano's comments imply, that the light loss within a microscopical preparation must be due to an extremely complicated set of factors; and this is a great difficulty with a method like that of the Caspersson school, in which the nonspecific light loss must be estimated, apparently by inference from measurements at wavelengths where there is no specific absorption. Perhaps Dr. Thorell will comment upon this part of Dr. Fano's criticism. In order to avoid this source of error, in each of our methods we have attempted to measure a blank designed to cancel out the non-specific light loss. Three of these methods involve adding specifically absorbing groups to the protein or nucleic acid already present. Only the blank in the method for determination of total nucleic acid involves removal of any considerable amount of the nuclear material; and we feel that for this reason it is probably somewhat less satisfactory than the other blanks.

In the usual photometric chemical analysis it is easy to tell whether a blank is adequate by checking the photometric analysis against a known concentration. A similar empirical calibration has been reported in our work, in which the microscopic determinations of total nucleic acid in a few nuclei were compared with gross analyses. Although the microscopic mean value is based upon a somewhat inadequate sample the comparison indicates that the error is not greater than 10% in this total nucleic acid method, which involves the use of the less satisfactory blank. We believe the error in the protein method may be much smaller than this.

THORELL: Errors in microspectrographic measurements can be regarded as deriving mainly from two sources. First, the error involved in the technical procedure of the light intensity measurements. This can be kept on a low level, and is in most cases about .01%. Thus, that value can be regarded as a measure of the reproducibility of a single measurement value. Second, there is the error in the calculated value of the substance being measured. That error is largely dependent on the nature and state of the material under investigation. Among the most important factors is the technique of the histological preparation, on which the optical conditions in the material mainly depend (refractive index boundaries, etc.).

The interference in quantitative light absorption analyses by the possible distribution of the absorbing substances in submicroscopic units can be considered according to the theories of Mie (1908, Ann. d. Phys. *25:* 377). (The formula of Raleigh, mentioned in the discussion with Dr. Pollister of my own paper, is a special case of Mie's theories, valid for infinitely small particles.) Calculations according to Mie's theories have been made by Caspersson (1932) for particles the size of which varies from 4λ

to 0.1λ. I will not enter into the details, but in general the calculations show that the risk of disturbing selective absorption is very small. (See Kolloid-Zeitschrift *60:* 151, 1932; *65:* 162 and 301, 1933). As Dr. Pollister mentioned, information about these factors can be obtained by means of measurements of the light losses at widely different values of λ.

Dr. Pollister's method is claimed to eliminate errors resulting from these unspecific light losses. However, the similarity between Dr. Pollister's method and the ordinary macrochemical photometrical procedure is, I think, somewhat overemphasized. Between the "blank" and the test is the not too gentle treatment with trichloracetic acid in the case of nucleic acid determination. A point in the case of the determination of protein is the calibration with test solutions; the results of this are then used for evaluating quantitative data from a very complicated and undefined medium, the tissue section.

THE COMPOSITION OF CHROMOSOMES DURING MITOSIS AND MEIOSIS

HANS RIS

I. The Composition of the Chromosomes in the Onion Root Tip during Mitosis

The techniques described in the preceding paper (Pollister and Ris) make it possible to analyze the nucleoproteins in chromosomes during cell division. The onion root tip is a classical object for the study of mitosis. Cells in all stages of division are easily obtained.

1. *Total nucleic acid*

The total nucleic acid content was determined on isolated nuclei in various stages of division. Root tips fixed in acetic-alcohol (1:3) were teased on a slide in 45% acetic acid. A number of cells were thus broken and the nuclei liberated. By letting this suspension dry the nuclei were fixed to the slide. The nucleic acid content of these nuclei is measured by determining the extinction of a known area a little larger than the nucleus within it. This area on the image plane is kept constant by closing the diaphragm on a cylinder of known diameter. As long as the nuclei lie within the area it does not matter what shape they have. The extinction values are then directly proportional to the nucleic acid content. In order to calculate the absolute amount of nucleic acid we have to know the extinction of a nucleic acid solution containing 1 mg/cc. This value ε is 20.0 if the thickness of the solution is 1 cm. If A is the area on the slide, the extinction of which is measured, expressed in cm, E the extinction at 2537 A corrected for structural light loss, then the nucleic acid content of one nucleus will be

$$NA = \frac{E}{\varepsilon} . A \text{ mg}$$

This method is however only valid when the area of absorbing substance is large compared with the non-absorbing area. The extinction values and absolute amounts are shown in the first column of Table 1. The values for metaphases were obtained on isolated metaphase plates still attached to the spindle. The spindle itself contains some nucleic acid, but insignificant amounts compared with the chromosomes. These data show that the total nucleic acid content of the nuclei increases during prophase and in the metaphase chromosomes is about twice as much as in the interphase nucleus. The values are averages of 3-5 nuclei.

2. *Desoxyribonucleic acid*

Cells in various phases of cell division were isolated on a slide as described above and stained with Feulgen. The extinction at 546 mμ is a measure of the relative amounts of desoxyribonucleic acid. The second column in Table 1 shows how the desoxyribonucleic acid increases in prophase to reach in metaphase about twice the amount of interphase nuclei. One daughter plate at anaphase is about one half of the metaphase plate as expected. Telophase is a little lower than anaphase for reasons which are not yet understood. The values in this column are averages of 5-10 nuclei.

3. *Nucleic acid-protein ratio*

The results of the nucleic acid determinations now raise the question whether there is an increase in the nucleic acid content of the chromosomes or whether there is an increase in the total mass of chromosomes. The answer is of course given by the ration nucleic acid/protein. On sections 5 μ thick the extinction of nuclei at 2537 A was measured. Then the slide was treated with trichloracetic acid-Millon reagent and the extinction at 3640 determined for the same nuclei. The ratios of E2537 to E3640 are

Table 1. Onion Mitosis

	Interphase	Early Prophase	Late Prophase	Metaphase	Anaphase	Telophase
	34.10^{-9} mg.	$34.5.10^{-9}$ mg.	36.10^{-9} mg.	65.10^{-9} mg.		
Total Nucleic acid	.060	.061	.063	.113		
Feulgen	.027	.041	.048	.071	.027	.022
Ratio Nucleic acid / Total protein (relative values)	2.2	2.2	2.3	[2.3]		
Ratio Nucleic acid / Resid. protein (relative values)	4.2	4.1	4.3			4.2
Residual protein	52%	54%	53%			
Ratio Pyronin / Methyl green	1.0		1.0	1.1		0.9
Ratio Protein / Pyronin	3.4	3.4	3.6			3.1

a relative measure for the nucleic acid/protein ratios and are given in Table 1, third column. They are the averages of 3-5 nuclei. The value for metaphase chromosomes was determined on isolated chromosomes on a smear. Since the ratio during prophase remains the same, we must conclude that both nucleic acid and protein increase in equal amounts.

4. *The relative amounts of histone and non-histone proteins*

If we determine, again on sections, the extinction at 2537 A and then remove the nucleic acid, treat the slide with sulfuric acid-Millon and determine the extinction of the same nuclei at 3640 A we get a relative measure for the nucleic acid/non-histone protein ratio. From this and the ratio nucleic acid/total protein we can calculate the relative proportions of the histone and non-histone protein fractions. From column four and five in Table 1 it is clear that the relative amounts of histone and non-histone protein remain the same during prophase.

5. *Relative amounts of ribonucleic acid and desoxyribonucleic acid*

Root tip sections, 5 μ thick, were stained with Unna's pyronin-methyl green mixture. The ratio of pyronin to methyl green as measured by the extinction at 550 mμ and 630 mμ was determined for nuclei in various stages of mitosis. As shown in column 6 of Table 1 the relative proportions of these two nucleic acids remain unchanged during mitosis.

6. *The protein-ribonucleic acid ratio*

This ratio can be determined by staining a section with pyronin and measuring the extinction at 550 mμ for nuclei in various stages of division. After destaining the slide is treated with the trichloracetic-Millon and the extinction of the same nuclei measured at 3640 A. This ratio also remains unchanged during mitosis as shown in the seventh column in Table 1.

The measurements reported here then indicate that during mitosis the composition of the chromosome does not change, but that the total mass of the chromosomes doubles during prophase.

II. The Composition of the Chromosomes during Meiosis in the Grasshopper

The large nuclei and chromosomes of grasshopper spermatocytes make them a favorite object for cytochemical studies. *Chorthophaga viridifasciata* was used in the studies reported below.

1. *Total nucleic acid*

To measure the amounts of nucleic acid nuclei of spermatocytes were isolated by teasing in 1% citric acid. The suspension was centrifuged and washed in 0.1% citric acid. This removes the cytoplasmic debris. The nuclei were then fixed in acetic-alcohol (1:3), spread on a slide and dried. The nucleic acid was then determined as described by Pollister and Ris in this volume. The first column of Table 2 contains the E values and absolute amounts of nucleic acid for nuclei in various stages. The numbers are averages of 3-5 nuclei. We see that in the first division the nucleic acid increases in prophase just as in the onion root tip cells. However there is no such increase in the second division, so that an early spermatid contains one fourth of the nucleic acid of the first spermatocyte metaphase.

2. *Desoxyribonucleic acid*

In the second column of Table 2 the E values for the Feulgen are recorded as determined on a smear. The desoxyribonucleic acid also doubles in the first prophase, but not in the second division.

3. *Nucleic acid-protein ratio*

This ratio was measured on sections 8 μ thick. It does not change appreciably during prophase (Table 2, column 3). This means that in prophase we have an increase in proteins as well as in nucleic acid.

Table 2. Grasshopper Meiosis

	Pre-leptotene	Leptotene	Pachytene	Metaphase I	Anaphase I	Interphase	Prophase II	Metaphase II	Spermatid 1	Spermatid 2
	23.10^{-9} mg.	28.10^{-9} mg.	41.10^{-9} mg.			24.10^{-9} mg.	20.10^{-9} mg.		11.10^{-9} mg.	
Total Nucleic acid	.085	.105	.155			.092	.072		.039	
Feulgen	.056	.061	.074	.097	.056	.041	.049	.046	.022	
Ratio Nucleic acid / Total protein (relative values)	1.3	1.3	1.1						1.6	1.2
Ratio Nucleic acid / Resid. protein (relative values)	3.0	3.0	2.5			2.9			3.6	5.4
Residual protein	43%	43%	44%						44%	22%
Ratio Pyronin / methyl green		1.8	1.6						1.6	1.1

4. *The relative amounts of histone and non-histone proteins*

From columns 3, 4 and 5 of Table 2 it is apparent that the relative quantities of histone and non-histone protein remain constant during meiosis. The non-histone protein amounts to 44%. In the late spermatid however a change takes place. The non-histone fraction decreases and the histone increases in proportion. This change in the composition of chromatin during spermatogenesis has been found in other organisms, for instance the trout sperm (Pollister and Mirsky, 1946). Since the work of Miescher it has been known that sperm nuclei contain almost exclusively protamines or histones. It is of interest that this same change can be shown cytochemically in the spermatogenesis of an insect.

5. *The relative amounts of ribonucleic acid and desoxyribonucleic acid*

Sections of grasshopper testis, fixed in acetic-alcohol, were stained with the pyronin-methyl green mixture and the relative amounts of the two dyes determined as described above. As the figures in Table 2 show, the relative amounts of these nucleic acids do not change during meiosis. In the late spermatid however there is a marked decrease in the relative amount of ribonucleic acid. This is expected from the decrease in non-histone protein, since the ribonucleic acid in chromosomes is combined with non-histone protein (Mirsky, this volume).

These data then indicate that during the meiotic divisions the relative composition of chromosomes remains the same. Only in the late spermatid is this composition altered. In the first prophase the total amount of chromosome substance increases about twofold, while in the second division it appears to remain constant from interphase to metaphase. The early spermatid then contains one fourth of the substance of the primary spermatocyte metaphase.

IV. Discussion

In the onion as well as in the grasshopper we measured the composition of the nuclei rather than the chromosomes. We might expect therefore that the measurements could be influenced by the composition of the nucleoplasm and the nucleolus. In the nuclei studied the nucleoplasm however can be only a very small fraction of the total mass. The nucleolus appears to be especially large in the onion root tip cells. Yet measurements show that its diameter is less than one fourth of the diameter of the nucleus. Its volume, therefore, is less than one sixty-fourth of the nuclear volume. We can thus safely neglect it.

Caspersson (1939) measured the nucleic acid content of grasshopper spermatocytes during prophase. He found an increase in nucleic acid in leptotene and suggested a relationship between the increase in desoxyribonucleic acid and gene-duplication. He mentions casually that the ratio of extinction at 2600 A to the extinction at 2750 A is the same in early leptotene and in metaphase chromosomes. This means then that the nucleic acid-protein ratio remains constant. Therefore, there seems to be no reason to emphasize especially the nucleic acid increase. In a later paper Caspersson (1941) proposed a generalized scheme for the changes in chromosome composition during mitosis. According to this the chromosomes at metaphase contain relatively more desoxyribonucleic acid, more histone and less non-histone proteins, and he compared these changes with those occurring during spermatogenesis. The measurements reported in this paper, while in general agreement with the data of Caspersson's (1939) paper, do not confirm his generalized conclusions and disagree particularly with his protein determinations.

The increase in nucleoproteins during prophase is of great interest. It can be interpreted in two ways: either it is the expression of chromosome and gene reproduction during prophase, or it signifies merely an increase in non-genic chromosomal substance or matrix. The gene material would then have to be a very small part of one of the fractions analyzed. The fact that all the known fractions of the chromosomes increase proportionately, that they are increased twofold and that no such increase occurs in the second meiotic division make it at present perhaps more likely that it is the actual result of chromosome reproduction during prophase.

Summary

The cytochemical methods described in the paper of Pollister and Ris were used to study the composition of chromosomes during mitosis in the onion root tip and meiosis in the grasshopper. It was found that the chromosome composition does not change during cell division. During the mitotic prophase and in the first meiotic prophase the constituents of chromosomes increase about twofold. No such increase takes place in the second meiotic division. From the study of isolated chromosomes (Mirsky, this volume) we know that chromosomes vary greatly in composition from tissue to tissue. During cell division within the same tissue, however, no change was observed. It remains to be seen whether in non-dividing and dividing cells of the same tissue any such difference exists.

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THE ACTION OF PROSTATE PHOSPHATASE ON YEAST NUCLEIC ACID[1]

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The application of enzymes as tools for the elucidation of the structure of nucleic acids[3] has been particularly successful in the field of desoxyribonucleic acids. Up to now the enzymatic technique of the hydrolysis of desoxyribonucleic acids, developed by Levene (1929) and by Thannhauser (1926-1935) and their associates, is the only method which permits the preparation of desoxyribose, desoxyribonucleosides and desoxyribonucleotides.

In the field of ribonucleic acid, enzymatic methods have so far not received the same degree of attention because it is possible to achieve the partial hydrolysis of ribonucleic acid to mononucleotides or nucleosides by ordinary laboratory reagents. Even in this field, however, all efforts of obtaining, by chemical methods, hydrolysis products of a higher degree of complexity than that of mononucleotides were completely unsuccessful. In 1917, Thannhauser made the first attempt to obtain such products by enzyme action. He reported the isolation of a trinucleotide containing adenylic, guanylic and cytidylic acid from the digestion of yeast nucleic acid with duodenal juice. The stage of enzyme chemistry at this period, as well as the difficulty of differentiating lower polynucleotides from mixtures of mononucleotides, impeded for a long time the further development of this work.

It is obvious, however, that the most important requirement for any further insight into the structure of ribonucleic acid is the availability of information regarding the nature of hydrolysis products of higher complexity than that of the mononucleotides. We resumed, therefore, the study of the hydrolysis of ribonucleic acid by enzymatic methods.

The investigation of the intermediary polynucleotides formed during the enzymatic hydrolysis of ribonucleic acid is possible only if this hydrolysis proceeds in experimentally distinguishable steps. It was, therefore, the first purpose of our work to find an enzyme system by which such a stepwise degradation of ribonucleic acid could be achieved. It was found in an earlier extensive study on alkaline phosphatase (Schmidt and Thannhauser, 1943) that this enzyme is not suitable for this purpose. Alkaline phosphatase splits ribonucleic acid rather slowly but completely into nucleosides and inorganic phosphate. The time curve of the action of alkaline phosphatase on ribonucleic acid is a smooth curve without any break which might indicate the accumulation of intermediaries at any phase of the reaction. We remarked in our paper that "alkaline phosphatase offers no simple possibilities for the structural differentiation of the various phosphorus radicals in ribonucleic acid."

In the present investigation we studied the action of ribonuclease and acid phosphatase from a similar point of view. Evidence for our conclusion, that the hydrolysis of ribonucleic acid by this enzyme system represents a stepwise degradation even before the stage of mononucleotides is reached, will be presented in this report. We shall also discuss certain inferences of our results regarding the mechanism of the enzymatic hydrolysis of ribonucleic acid.

Before going into the discussion of the experiments, it seems useful to review briefly some aspects of the structure of ribonucleic acid as far as they have a bearing on the interpretation of our observations.

The conception of the tetranucleotide structure of the nucleic acids is chiefly based on two facts: first, not more than 4 different component nucleotides have been isolated from individual nucleic acid samples prepared from a great variety of animals and plants; second, the 4 mononucleotide groups have been found to occur in equivalent proportions in all nucleic acid samples which until now have been analyzed with quantitative methods. In the field of the ribonucleic acids, such quantitative data have been available until recently for a very small number of nucleic acid specimens, chiefly for yeast and triticonucleic acid. Schneider (1946), however, reported the results of a study in which the ribonucleic acid content of various animal tissues was determined by the use of two independent methods namely that of Schneider (1945) which is based on pentose determination in the nucleic acid fraction, and that of Schmidt and Thannhauser (1946) which is based on the determination of the total ribonucleic acid P. It was found that the molecular ratio of pentose to total ribonucleic acid P was close to 0.5

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[3] While this paper was in print the authors received the news of the death of John Masson Gulland. They wish to introduce their study with the acknowledgement of Dr. Gulland's work and his stimulating and constructive influence on all recent investigations concerned with the structure of nucleic acids. His last admirable contribution, the synthesis of uridine-2-phosphoric acid, represents a new and important approach to this problem with the methods of synthetic organic chemistry.

in all investigated tissues. Schneider's results were independently confirmed by Schmidt and Thannhauser in a series of unpublished experiments. Since only the pentose groups of the purine nucleotides react in the procedure used by Schneider the inference is that equimolecular amounts of purine and pyrimidine nucleotides are present in the ribonucleic acid fractions of all analyzed animal tissues. This finding, of course, does not give any information concerning the distribution and mutual proportions of the individual purines and pyrimidines within the RNA molecules of various tissues. It should be emphasized, moreover, that the comparison between both methods has been limited until now to animal tissues. Nevertheless, the identity of the purine: pyrimidine ratio in the ribonucleic acid fractions of various animal tissues represents a remarkable analogy to the apparent identity of the proportions of the component nucleotides of desoxyribonucleic acid in various tissues. As long as it was believed that the molecular weights of the nucleic acids had the minimum values consistent with the results of chemical analysis these results could be interpreted only by the classical tetranucleotide formula.

Recent observations, however, lead uniformly to the conclusion that the molecular weights of the nucleic acids have much higher values than those of tetranucleotides (Cohen and Stanley, 1944). Thus the question arises as to whether nucleic acids are polymers of tetranucleotides or whether the individual mononucleotide groups are distributed in a less regular manner throughout the nucleic acid molecule (Loring, 1947).

Further information regarding the structure of nucleic acids, particularly that of ribonucleic acid, can be obtained, therefore, only by methods which would permit the cleavage of the nucleic acid molecule into small fragments consisting of more than one mononucleotide group and by subsequent isolation and analysis of such fragments. At present the only agent known to effect this type of cleavage of ribonucleic acid is ribonuclease. Despite the fact that Loring and Carpenter (1943) were recently able to demonstrate the appearance of mononucleotides as end products of ribonuclease action, it is generally assumed that a large portion of the splitproducts are polynucleotides of relatively low molecular weights.

The fractionation of the polynucleotide mixtures resulting from the action of ribonuclease on ribonucleic acid appears to be a formidable task. Any consideration of this problem recalls the long and fruitless efforts of investigators of an earlier period of nucleic acid chemistry who tried to isolate and to characterize polynucleotides from hydrolysis mixtures of nucleic acids. One of the greatest difficulties of the analysis of such mixtures is the distinction between polynucleotides of low molecular weight and mixtures of mononucleotides (Jones, 1920; Levene, 1931).

It occurred to us that this difficulty might be obviated by selectivity removing the mononucleotides and the phosphomonoester groups by phosphatase action. Even if the attempted partition of the phosphatases into phosphomono- and diesterases by Uzawa (1932) has been shown to be untenable by later authors, particularly by Klein and Rossi (1935), it is certain that animal phosphatases hydrolyze phosphomonoesters many times more rapidly than diesters. Schmidt and Thannhauser (1943) have shown that the rate of hydrolysis of monophenyl phosphate by intestinal phosphatase is approximately 140 times greater than that of diphenylphosphate. This behavior is probably the explanation for the long known fact that nucleic acids are hydrolyzed much more slowly by phosphatases than are the mononucleotides.

The lack of information on the metabolic behavior of phosphoric acid diesters is at present the most important gap in our knowledge of the metabolism of phosphorus compounds since a large part of the organic phosphorus compounds of the cells are diesters such as nucleic acids and phospholipids. We know very little about the enzymes involved in the cleavage of phosphoric diester linkages and nothing about the biological synthesis of phosphoric diesters. The wealth of information on enzymatic phosphorylations concerns exclusively the formation of phosphoric monoesters, phosphoamides or pyrophosphates.

Takahashi (1932) and Gulland and Jackson (1940) have made use of the different behavior of mono- and diesters of phosphoric acid against enzymes in order to obtain information regarding the nature of the phosphoric acid linkages in the RNA molecule. Bolomey and Allen found in 1942 that the action of acid phosphatase from sweet almonds on ribonucleic acid was strongly enhanced by a preliminary incubation of ribonucleic acid with ribonuclease presumably because of the transformation of phosphoric acid diester groups into monoester groups by ribonuclease. The basis for this interpretation was an earlier investigation of Allen and Eiler (1940) who found that the action of ribonuclease was accompanied by the liberation of acid groups of the substrate.

The quantitative interpretation of the results of these investigations is difficult mainly because of the fact that rather weak phosphatase preparations were used. In consequence, very long incubation periods were necessary which made it difficult to avoid the interference of contaminating enzymes and other factors such as kinetic peculiarities which are often encountered in the action of phosphatase. For this reason we studied the action of highly potent samples of acid phosphatase from human prostate glands on ribonucleic acid.

MATERIALS

Ribonucleic acid was used in the form of its sodium salt, supplied by the Schwartz Laboratories. It contained 8.1% P. The N:P ratio was 15.7 : 4

(theoretical 15 : 4); the ratio of purine N to total N was 0.67. Ninety per cent of the total P precipitated when 2 cc. of a solution containing 15.4 mg. nucleinate were mixed with 10 cc. of glacial acetic acid.

Crystalline ribonuclease was prepared according to Kunitz (1940) and recrystallized three times from ammonium sulfate solution.

Crude solutions of acid phosphatase were obtained by homogenizing surgically enucleated hypertrophic prostate glands with 5 volumes of water in the Waring blender. The material was left overnight in the refrigerator after the addition of toluene and was then centrifuged. The supernatant was used for the experiments. (The surgical material was obtained through the kindness of Dr. J. Fishmann,

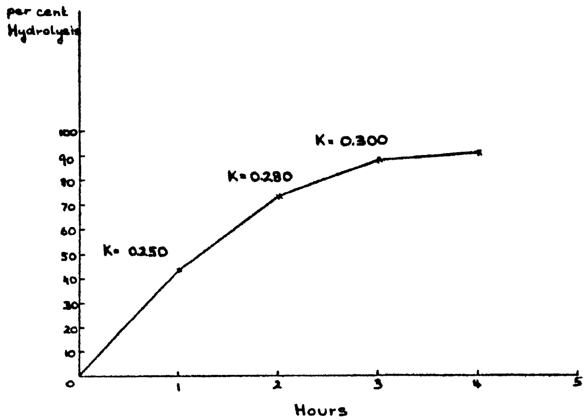

FIG. 1. Hydrolysis of 3-adenylic acid by prostate phosphatase. Total volume 13 cc. containing 1 cc. crude prostate gland extract 1: 500; 10 cc. N/10 acetate buffer pH 5.3; 50 mg. 3-adenylic acid, neutralized with NaOH.

$$K = \frac{1}{t\ (\text{hours})} \times \log \frac{a}{a-x}$$

Dr. F. Lipmann, and the staff of the Department of Urology, Massachusetts General Hospital, Boston.)

HYDROLYSIS OF MONONUCLEOTIDES BY PROSTATE PHOSPHATASE

The substrate solutions (3-adenylic acid, 3-guanylic acid) were brought to pH 7 by the addition of N sodium hydroxide or of N acetic acid. Two cc. of the solutions containing amounts equivalent to 50 mg. adenylic acid were mixed with 10 cc. N/10 sodium acetate buffer (pH 5.3). The mixture was warmed to 37° C and incubated with 1 cc. of dilute phosphatase solution (usually corresponding to 0.01 cc. of the original extract).

It was found that mononucleotides (3-adenylic and 3-guanylic acids) were very rapidly hydrolyzed under these conditions. Fifty mg. of either of the nucleotides were practically completely dephosphorylated within 3 hours by 0.01 cc. of the prostate extracts. The rates of hydrolysis of the mononucleotides are approximately three times higher than that of β-glycerophosphoric acid.

The time curves of the hydrolysis of the mononucleotides follow the course of a unimolecular reaction up to a degree of hydrolysis of 80%. This indicates that the enzymatic dephosphorylation of the mononucleotides by prostate phosphatase is very slightly influenced by the accumulation of the hydrolysis products (Fig. 1).

BEHAVIOR OF ACID PHOSPHATASE AGAINST RIBONUCLEIC ACID

Stability of ribonucleic acid at the pH optimum of prostate phosphatase

It was found that ribonucleic acid is stable when incubated for several days at 37° C at a pH of 5.3.

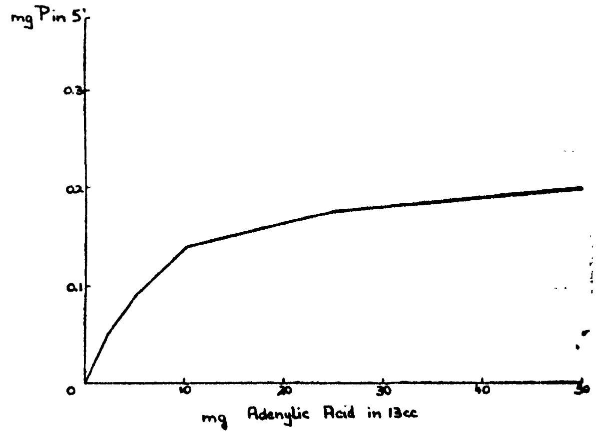

FIG. 2. Influence of the adenylic acid concentration on the rate of the enzymatic hydrolysis of adenylic acid by 1 cc. prostate extract 1: 500. General conditions as in Fig. 1.

This was concluded from the fact that the yield of the precipitate obtained with 5 volumes of glacial acetic acid (90% of the total P) did not change when the ribonucleic acid was incubated for 2 days at pH 5.3 under conditions comparable with those of the enzyme experiments.

Action of prostate phosphatase on ribonucleic acid

The lower curve in Fig. 3 was obtained by the incubation of 0.01 cc. of prostate phosphatase with an amount of ribonucleic acid equimolecular (as tetranucleotide) with that of 3-adenylic acid used in the previous experiments. It can be seen that only very small amounts of inorganic phosphorus are formed even after an extended period of incubation. The resulting slow rate of the enzymatic hydrolysis by prostate phosphatase cannot be explained by an inhibitory effect of ribonucleic acid on acid phosphatase since it could be shown that the amounts of P formed by acid phosphatase from adenylic acid were practically identical with those obtained after the enzymatic digestion of a mixture of adenylic acid and ribonucleic acid (Table 1).

The release of the minute amounts of phosphate

under the conditions just described must be attributed nevertheless to enzyme action since considerable rates of dephosphorylation are observed when very large amounts of prostate extracts (1 to 10 cc.) are used (Fig. 4). Since it is easy to demonstrate the presence of ribonuclease in all crude extracts from prostate glands it cannot be decided as yet whether or not prostate phosphatase is able to hydrolyze ribonucleic acid in the absence of ribonuclease.

TABLE 1. ENZYMATIC DEPHOSPHORYLATION OF ADENYLIC ACID IN PURE SOLUTION AND IN PRESENCE OF RIBONUCLEIC ACID

Substrate	Hydrolysis after 30 hours incubation with 0.01 cc. prostate phosphatase	
	mg. P	% hydrolysis
50 mg. adenylic acid	1.62	53
50 mg. adenylic acid+200 mg. ribonucleic acid	1.67	55

Schmidt and Thannhauser (1943), as well as Zittle (1947), demonstrated that ribonucleic acid can be dephosphorylated by alkaline phosphatase in the absence of ribonuclease. Under suitable conditions (small amounts of substrate, relatively large amounts of enzymes), the total amount of ribonucleic acid P may be converted to inorganic P by the action of intestinal phosphatase. It appears that intestinal phosphatase possesses diesterase activity in addition to its power of hydrolysing phosphoric acid monoesters although the former is approximately 140 times weaker than the latter.

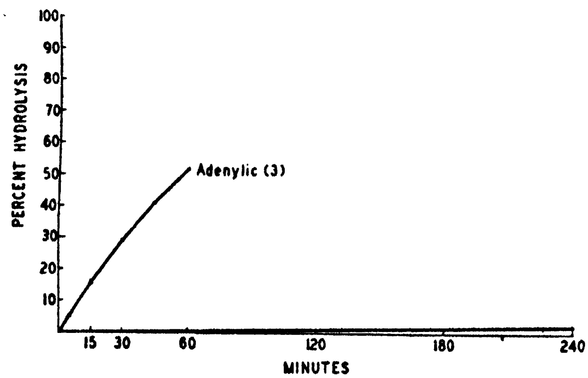

FIG. 3. Hydrolysis of ribonucleic acid (lower curve) and 3-adenylic acid (upper curve) by prostate phosphatase. Conditions as described in Fig. 1. The sample with ribonucleic acid contained 200 mg. yeast nucleic acid.

Action of prostate phosphatase on ribonucleic acid after incubation with ribonuclease

The behavior of acid phosphatase against ribonucleic acid changes strikingly when ribonucleic acid is digested with ribonuclease prior to the incubation with acid phosphatase. After digestion with ribonuclease, ribonucleic acid is dephosphorylated by phosphatase at an initial rate which closely approaches that of the hydrolysis of adenylic acid (Fig. 5). This rapid dephosphorylation continues until the amount of inorganic P formed amounts to 20-25% of the total P of the nucleic acid. At this stage, the time curves exhibit a distinct break because, the rate of the dephosphorylation becomes much slower (Figs. 5-7).

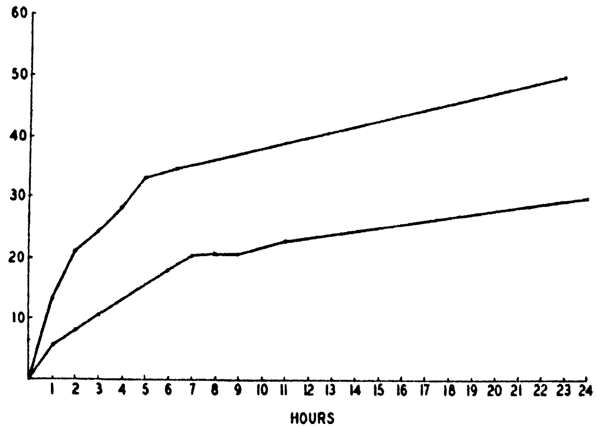

FIG. 4. Hydrolysis of ribonucleic acid by large amounts of crude prostate extracts.

Total volume 26 cc. containing 200 mg. sodium ribonucleate and 10 cc. acetate buffer pH 5.3.
lower curve: with 3 cc. prostate extract 1: 5.
upper curve: with 10 cc. prostate extract 1: 5.

The enhancement of the action of phosphatase on ribonucleic acid by a preliminary digestion of the substrate with ribonuclease has already been demonstrated by Bolomey and Allen (1942). These authors, however, did not investigate the quantitative aspects of this effect.

The striking initial increase of the enzymatic dephosphorylation of ribonucleic acid after its treatment with ribonuclease suggests the assumption that ribonuclease transforms phosphoric acid diester linkages which are known to be rather resistant against phosphatase into monoester linkages which are rapidly hydrolyzed by this enzyme. This explanation is supported by earlier observations of Allen and Eiler (1940) who found the action of ribonuclease on ribonucleic acid is accompanied by the appearance of acidic groups in the digest in amounts corresponding to one equivalent of acid per 4 to 5 phosphoric acid groups.

The "break" in the time curves of the enzymatic dephosphorylation of ribonuclease-treated ribonucleic acid at a stage of 20 to 25% hydrolysis is very characteristic. It could be demonstrated that the addition of new phosphatase at this stage failed to increase appreciably the rate of the dephosphorylation. On the other hand, addition of a mononucleo-

tide such as 3-adenylic acid in amounts equivalent to one-fourth of the total ribonucleic acid P immediately restored the rate of the formation of inorganic P to the high initial value.

These observations exclude the possibility that the "break" was caused by an inactivation or inhibition of the phosphatase. They suggest the assumption that the reasons for the break were the structural properties of the substrate. This assumption was supported further by the observation that the stage of hydrolysis (20 to 25%) at which the break occurred remained unchanged when different amounts of ribonucleic acid (ranging between 50 and 200 mg.) were incubated with the same amount of phosphatase in the same total volume or when the amount of enzyme used was increased 100-fold under otherwise unchanged conditions (Fig. 7).

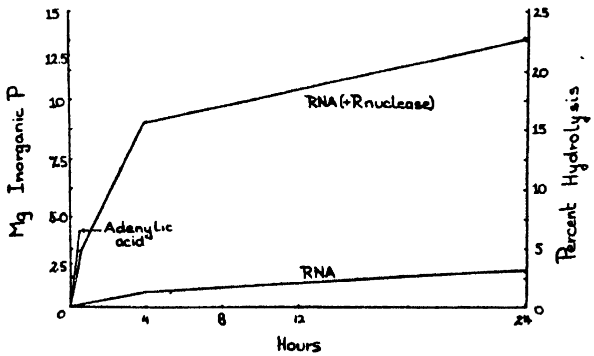

FIG. 5. Hydrolysis of ribonucleic acid by prostate phosphatase without and with preliminary incubation with ribonuclease (RNA + ribonuclease). Hydrolysis of adenylic acid under the same conditions (adenylic). Conditions as described in Fig. 2.

The ribonucleate solution of the curve RNA + ribonuclease was incubated for 24 hours with 1.2 mg. crystallized ribonuclease prior to the incubation with prostate extract. The percentage of hydrolysis on the right ordinate applies exclusively to the curves for RNA.

In order to decide whether the inorganic phosphate which was formed during the first rapid phase of the dephosphorylation originated from the various nucleotide groups or from a specific nucleotide group of the substrate, we investigated the mutual proportion of the different nucleotide groups in the substrate before and after the rapid phase of the dephosphorylation. So far, we have limited our studies to the partition of the nucleotide groups into purine and pyrimidine nucleotides.

METHOD

The different stabilities of the purine and pyrimidine nucleotides against mineral acids at 100° C offered a suitable basis for their quantitative partition.

It could be demonstrated that the time curves of hydrolysis of ribonucleic acid in sulfuric acid solutions between normalities of 0.5 and 2, while differing from each other in respect to the rates of hydrolysis, reached their asymptotic phases without exception when the hydrolysis had reached a degree of 50% of the total P. At a given concentration of sulfuric acid, the concentration of ribonucleic acid had only a negligible influence on the degree of hydrolysis at this phase. The same was demonstrated for inorganic phosphate in the range of the concentrations occurring in the experiments of this investigation.

It could be demonstrated that the amounts of the "60′ P" represent quantitatively the purine nucleotide P of the hydrolyzed ribonucleic acid. The necessary amounts of hydrolysate were obtained by hydrolyzing six 2 cc. aliquots of a ribonucleate solution containing 200 mg. sodium ribonucleate in 26 cc. under the conditions described in the following chapter. The hydrolysate was divided into two equal parts, A and B. Part A was used for the determinations of total N and of the purine N (determined as the total N of the purine silver compounds after precipitation of the acid hydrolysate with silver sulfate). Part B was treated as follows: The sulfuric acid was removed by adding a hot saturated solution of barium hydroxide to the hot hydrolysate. An excess of barium hydroxide was carefully avoided. The result of the total P determinations in the barium filtrate proved that no appreciable loss of nucleotides occurred during the precipitation of the barium sulfate.

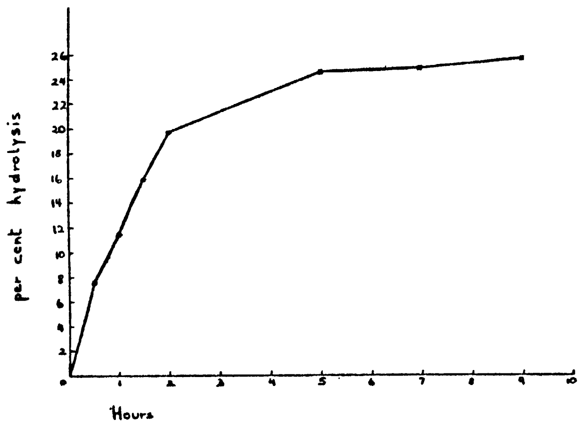

FIG. 6. Initial stage of the hydrolysis of ribonucleic acid by prostate extracts after preliminary incubation with ribonuclease. Conditions as in Fig. 2.

$$\% \text{ hydrolysis}: \frac{\text{mg. inorganic P} \times 100}{\text{mg. total RNA P}}$$

The filtrate from the barium sulfate was precipitated with a 25% solution of neutral lead acetate after the addition of 1/10 volume of a molar solution of primary sodium phosphate and after neutralization to pH 6. Under these conditions all P compounds are removed from the filtrate. After the

removal of the lead, the filtrate was acidified by adding one-tenth volume of 5 N sulfuric acid. The total N was determined in an aliquot of the acidified filtrate. Another aliquot was precipitated, while being heated, with a hot saturated solution of silver sulfate. After standing overnight the silver precipitate was centrifuged off, washed and decomposed by hydrochloric acid. The total N was determined in an aliquot of the decomposition liquid of the silver precipitate.

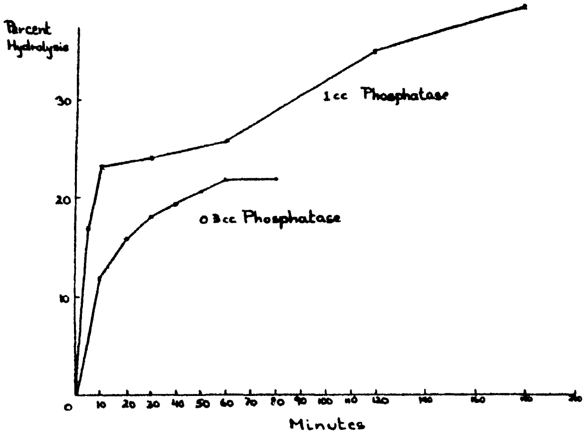

FIG. 7. Hydrolysis of ribonucleic acid by large amounts of prostate extracts after preliminary incubation with ribonuclease. General conditions as in Fig. 4 and 5.

Lower curve: 0.3 cc. prostate extract 1: 5
Upper curve: 1 cc. prostate extract 1: 5

It was found that the total N content of the hydrolysate after the quantitative removal of the nucleotides was practically identical with the purine N of the hydrolysate. This means that the acid hydrolysis did not affect the phosphorus groups of the pyrimidine nucleotides. Since the amount of inorganic P liberated is equivalent to one-fifth of the purine N it represents quantitatively the amount of P present in the form of purine nucleotides in the hydrolyzed sample of ribonucleic acid.

It should be emphasized that these observations concerning the behavior of ribonucleic acid during the hydrolysis with dilute mineral acids are not new, but confirm earlier findings of Jones (1920) with a more elaborate technique. This author concluded from the surprising similarity between the course of hydrolysis of ribonucleic acid and that of a corresponding mixture of the mononucleotides that the phosphorus groups did not participate in the interlinkage between the mononucleotides in the nucleic acid molecule. Our time curves, however, demonstrate the presence of a short initial lag period of the acid hydrolysis of ribonucleic acid in the range of the lower acid concentrations. This lag period may be interpreted as the time required for the hydrolysis of the highly acid sensitive interlinkages between the mononucleotides.

PROCEDURE FOR THE QUANTITATIVE PARTITION OF RIBONUCLEOTIDES INTO PURINE AND PYRIMIDINE NUCLEOTIDES

An aliquot of the nucleotide solution containing (sample A) is pipetted into a wide Pyrex test tube. The volume of the solution is brought to exactly 5 cc. by the addition of water. After the addition of 5 cc. of 3 N sulfuric acid the test tube is immersed in a strongly boiling water bath. As a control, an aliquot of a solution of ribonucleic acid which had not been exposed to the action of the phosphatase is

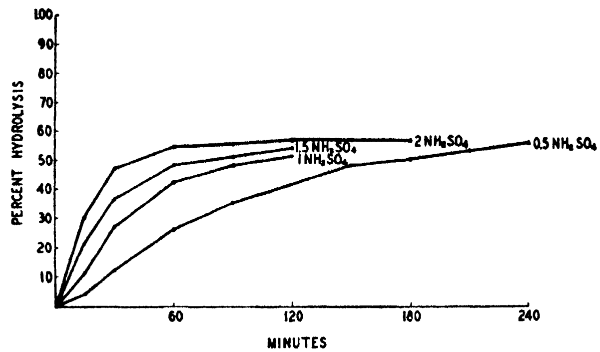

FIG. 8. Acid hydrolysis of yeast nucleic acid in sulfuric acid solutions of different concentrations at 100° C.

treated exactly in the same way and immersed in the boiling water bath simultaneously with the sample A. After one hour both samples are placed into a large beaker of cold water. After cooling, the amounts of total and inorganic P are determined in both samples. The amounts of inorganic P after hydrolysis of the control are 50% ± 5% of those of the total P.

Table 2 shows the results of an experiment in which the hydrolysis method outlined above was

TABLE 2.

1	2	3	4	5	6
Sample	Inorganic P formed during the incubation		Inorganic P formed formed after hydrolysis		
	mg.	per cent of the total P	mg.	per cent of the total P	mg. P(4) − mg. P(2)
A	0	0	6.9	48.1	6.9
B	0	0	6.9	48.1	6.9
C	2.5	17.85	9.6		7.1

A. 4 cc. sodium ribonucleate solution (containing 200 mg.) +20 cc. sodium acetate buffer (pH 5.3)+2.1 cc. water incubated for 28 hours at 37° C.
B. Substrate-Buffer mixture as in A; incubated for 28 hours with 1.2 mg. crystalline ribonuclease (dissolved in 0.1 cc. water).
C. Substrate-Buffer-Ribonuclease mixture as in B. After 24 hours incubation, 1 cc. prostate phosphatase (corresponding to 0.01 cc. crude extract) is added. The incubation is continued for 4 hours.

applied to a sample of yeast nucleic acid which had been preincubated for 24 hours with ribonuclease and which was then incubated with 0.01 cc. of acid prostate phosphatase for 4 hours. It can be seen that the amount of the hydrolyzable fraction of the organic P formed during the incubation with phosphatase originated exclusively from the pyrimidine nucleotides.

DISCUSSION

The selective hydrolysis of pyrimidine nucleotide groups during the rapid phase of phosphatase action on "depolymerized" ribonucleic acid can hardly be attributed to the specificity of acid phosphatase since it has been demonstrated that the purine nucleotides are hydrolyzed by this enzyme with great rapidity. Since the stage of the hydrolysis at which the "break" occurs is independent of the substrate concentration or the enzyme concentration, it is reasonable to connect the "break" with a structural difference between the organic phosphorus groups of the substrate. Thus, the striking enhancement of the initial rate of the phosphatase action by preliminary incubation of ribonucleic acid with ribonuclease, and the fact that this enhancement involves exclusively one-half of the pyrimidine nucleotide groups can only mean that the action of ribonuclease involves specifically, or at least preferentially, one of the two pyrimidine nucleotide groups.

The striking difference between the rates of hydrolysis of genuine ribonucleic acid on the one hand and those of "depolymerized" ribonucleic acid and mononucleotides on the other hand strongly suggests the assumption that the action of ribonuclease consists in the transformation of phosphoric diester or anhydride groups into phosphoric monoester groups. This interpretation is in agreement with earlier observations of Allen and Eiler (1940) that the action of ribonuclease is accompanied by the formation of new acid groups amounting to one equivalent for each four or five nucleotide groups.

The specificity of ribonuclease for one of the pyrimidine nucleotide groups furnishes a plausible interpretation for the observation why the action of this enzyme does not result in the complete degradation of ribonucleic acids to mononucleotides. However, any further inference as to the nature of the products of the action of ribonuclease will have to await additional experimental information.

SUMMARY

1. Prostate phosphatase hydrolyses ribonucleic acid at a very low rate, if at all.

2. After a preliminary incubation of ribonucleic acid with ribonuclease, 20 to 25% of its phosphoric acid groups are rapidly hydrolyzed by prostate phosphatase. The rate of hydrolysis under these conditions is similar to that of the hydrolysis of mononucleotides. The percentage of the ribonucleic acid P which is rapidly hydrolyzed by phosphatase after preliminary incubation of ribonucleic acid with ribonuclease is independent of the concentrations of ribonucleic acid and of those of phosphatase.

3. The action of ribonuclease involves specifically or at least preferentially one of the pyrimidine nucleotide groups of ribonucleic acid.

4. A hydrolysis method for the quantitative partition of ribonucleotides into purine and pyrimidine nucleotides has been developed.

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Discussion

FRIEDENWALD: In connection with the discussion on the action of phosphatases on nucleic acid it may be of interest to report some work by Miss Crowell and myself on the use of nucleic acids as substrates for the histochemical phosphatase reaction. We have used the Gomori technique for alkaline phosphatase with the modification that the reaction was performed on fresh frozen sections of tissue instead of on fixed and paraffin embedded sections. This modification results in some loss of the crispness of the histological picture, since the sections are 25 to 30 micra thick, but avoids at least any such extensive denaturation and inactivation as is entailed in the original procedure.

Using this technique on rat's kidney it was found that yeast nucleic acid yielded a phosphate deposit both in the nucleus and in the cytoplasm of the cells. Highly viscous preparations of thymonucleic acid obtained from rabbit liver failed to yield any phosphate deposit, but the same preparations partially depolymerized by boiling in acid yielded phosphate deposit in the nucleus but not in the cytoplasm. It would appear that some components of the enzyme system necessary for the dephosphorylation of thymonucleic acid are absent in the cytoplasm.

Applying this technique to the rat's cornea we at first obtained no phosphate deposit from either substrate. Since the number of cells per section in the rat's cornea is very much smaller than that per section in the rat's kidney, it appeared that some factor necessary for the reaction might be diluted out by our substrate solution. We therefore added various possible cofactors and found that on addition of very small amounts of KCl and glucose a heavy deposit of phosphate in the tissue was obtained. As in the case of the kidney, yeast nucleic acid yielded phosphate deposit in both cytoplasm and nucleus, while the partially degraded thymonucleic acid yielded a deposit only in the nucleus.

The role of potassium in this reaction appears to be specific and not simply a salt effect, since the substrate solution without potassium contains relatively large amounts of calcium chloride and barbital buffer. Moreover the potassium cannot be replaced by sodium or magnesium. The role of potassium in certain phosphate transferring systems is well known. The role of glucose in our reaction remains obscure. Energy should not be required for the decomposition of ester phosphate bonds and the tissue itself contains glycogen. Dr. Greenstein this morning referred to the possibility that the difference between the results with dialyzed and undialyzed tissue homogenates might be explained by the presence in the undialyzed preparation of a phosphate acceptor. It might be that glucose in our experiments provides such an acceptor. At any rate it would appear that the dephosphorylation of nucleic acid in frozen tissue sections is a more complicated process and perhaps more intimately related to other enzymic activities than it is in tissue homogenates.

KURNICK: It appears that the phosphatases are inactive or only very slightly active against native (polymerized) nucleic acids. Isolated, unfixed chromosomes contain a high concentration of alkaline phosphatase as determined quantitatively on a glycerophosphate substrate. However, no inorganic phosphorus is liberated, in controls, from their native desoxy- and ribosenucleic acids. Since all the phosphatase is localized in the residual ribonucleoprotein which remains after extraction with 1M NaCl, the phosphatase activity is even higher in this chromosomal fraction. Here again, incubation without the glycerophosphate buffer releases inorganic phosphorus only after many hours (at alkaline pH), at which time much RNA has passed into solution and is dialyzable.

It is possible that the very slow release of phosphorus from ribonucleic acid by the crude phosphatase extracts prepared from prostatic tissue, as observed by Dr. Schmidt, may be due to a small admixture of ribonuclease extracted simultaneously with the phosphatase, rather than to the latter alone.

In the case of histochemical studies using nucleic acids as substrates, such as those presented by Dr. Friedenwald, it is probable, therefore, that what is determined is not a specific phosphatase, but the coexistence of a specific nuclease and of alkaline phosphatase. Both desoxyribonuclease and ribonuclease liberate a small amount of mononucleotides. These are acted upon by phosphatase without apparent specificity. Therefore, it would appear that one should speak of specific nucleases rather than of specific phosphatases. Thus, Dr. Friedenwald's findings could be interpreted as indicating the presence of desoxyribonuclease in the nucleus, of ribonuclease in both nucleus and cytoplasm, and of phosphatase in both sites. The marked reduction in "phosphatase" activity on nucleic acid substrates after alcohol fixation is probably due to the inactivation of the nucleases by the alcohol. If the nucleic acids are pre-treated with depolymerase, thus yielding the susceptible nucleotides, the alcohol treated tissues again demonstrate phosphatase activity upon these substrates.

ZITTLE: Dr. Schmidt, will you discuss what you have found concerning the hydrolysis products of ribonuclease action and the report of Loring and Carpenter (J. Biol. Chem. *150:* 381, 1943) that the four mononucleotides could be isolated from the hydrolysate. Several workers agree that the extent of hydrolysis is about 25%, but there is this disagreement as to the products of hydrolysis.

SCHMIDT: The question is certainly justified, as the formation of larger amounts of all four mononucleotides by ribonuclease action is in contradiction with our observations. The explanation of this discrepancy will be possible only after the isolation of the split product formed under our conditions.

NUCLEIC ACIDS IN NORMAL AND NEOPLASTIC TISSUES

WALTER C. SCHNEIDER[1]

The study of the distribution and the metabolism of the nucleic acids has been hampered by the lack of adequate chemical methods for the estimation of these compounds. Within the last three years, however, several methods have been proposed for the determination of these compounds and it was considered desirable to review the methods and their relative merits prior to the presentation of some of the results which have been obtained.

It is now generally accepted that nucleic acids fall into two general classes: the desoxypentose nucleic acids (DNA) and the pentose nucleic acids (PNA), all the representatives of which presumably contain d-desoxyribose and d-ribose respectively. In addition to the difference in the carbohydrate components, PNA contains the pyrimidine base, uracil, while DNA contains methyl uracil.

In pure solutions quantitative measurement of the nucleic acids is comparatively simple. The total nucleic acid content can be obtained by measurement of the organic phosphorus in the solution, by estimation of the purine nitrogen in the solution, or by determination of the absorption of the solution at 2600 A (a measure of the purine and pyrimidine content). Differentiation of the nucleic acids can most easily be obtained by color reactions for the carbohydrate components. DNA can be estimated from the intensity of colors produced with diphenylamine (Dische, 1930), carbazole (Dische, 1930), tryptophane (Cohen, 1944) or cysteine (Dische, 1944). PNA can be measured from the intensity of the colors produced with orcinol (Mejbaum, 1939) or phloroglucinol (Euler and Hahn, 1946) or by measurement of the furfural produced after hydrolysis of the nucleic acid (Brachett, 1941; Davidson and Waymouth, 1944a). It should be pointed out that although both nucleic acids react with carbazole and orcinol (Schneider, 1945a), these methods are extremely useful because they possess greater sensitivity than the other more specific reactions. In addition, carbazole reacts only with the pyrimidine-bound carbohydrates of DNA, while diphenylamine and cysteine react only with the purine-bound carbohydrates (Dische, 1930, 1944; Schneider 1945a). Tryptophane and orcinol react with all of the carbohydrate groups of DNA (Cohen, 1944; Schneider, 1945a).

The quantitative measurement of any compound usually requires the availability of the compound in pure form to serve as a primary standard. In the case of the nucleic acids, the composition often varies considerably in different samples depending upon the method of preparation, the source, and even in different samples prepared from the same tissue by the same method. Thus it would appear that the available nucleic acids would be unsuitable as primary standards. It was found, however, that nucleic acids of varying degrees of purity, prepared by different methods from a variety of sources, produced the same color intensity in the diphenylamine, carbazole and orcinol reactions if the color intensity was referred to the phosphorus content of the samples (Schneider, 1945a). Thus, impure nucleic acids can be used as primary standards in the colorimetric reactions, provided the color intensities produced are related to the phosphorus content of the nucleic acid.

In the determination of the nucleic acids in biological material, another requisite must be met; methods must be devised to extract the nucleic acids quantitatively from the tissue and free from contaminants which might interfere with the analytical procedures. Davidson and Waymouth (1944a) extracted animal tissues with 10% NaCl after acid-soluble compounds and lipids had been extracted. The nucleic acids were then precipitated from the NaCl extract as lanthanum salts, and after removal of lanthanum, DNA was measured by the diphenylamine reaction and PNA was estimated by hydrolysis and colorimetric furfural determinations. Unfortunately, both the NaCl extraction and the lanthanum precipitation of the nucleic acids proved to be incomplete (Davidson and Waymouth, 1944b).

Schneider (1945a) reported the quantitative extraction of the nucleic acids from animal tissues by heating the tissue with trichloroacetic acid at 90° C after acid-soluble phosphorus compounds and phospholipids had been removed. DNA was estimated by the diphenylamine and carbazole reactions and PNA by the orcinol reaction. The total nucleic acids found by the colorimetric reactions accounted entirely for the phosphorus present in the nucleic acid extract. The quantitative nature of the hot trichloroacetic extraction was indicated by the complete removal of DNA from purified thymus nucleohistone and by the inability to increase the yield of nucleic acids from tissues either by repeated heating with trichloroacetic acid or with NaOH. Schramm and Dannenberg (1944) had independently found that PNA was quantitatively removed from tobacco mosaic virus by heating the virus with trichloroacetic acid.

At the same time as the trichloroacetic acid ex-

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traction of the nucleic acids was described, Schmidt and Thannhauser (1945) reported the determination of DNA and PNA in animal tissues by a new method. In this method acid-soluble compounds and lipids were removed and the tissue was treated at 37° C with 1 N NaOH. At the end of about 12 hours, the tissue had dissolved completely and the solution was acidified to precipitate protein and DNA. The filtrate contained PNA and inorganic phosphorus liberated from phosphoprotein. Determination of the total and inorganic phosphorus in the filtrate and of the total phosphorus present in the solution of the tissue in alkali permitted the calculation of DNA, PNA, and phosphoprotein phosphorus. Although it was shown that DNA, PNA and casein could be added to the tissue and recovered quantitatively, no attempt was made to show that the alkali treatment would quantitatively release PNA from nucleoproteins. The outstanding feature of the method which should be emphasized was that it permitted the quantitative separation of PNA and DNA, a feature of considerable importance in isotopic tracer studies.

The results obtained by Schneider (1945a) and by Schmidt and Thannhauser (1945) were in good agreement but some discrepancies did exist. Accordingly, the two methods were compared on the same sample of tissue. Six different rat tissues were treated to remove acid-soluble compounds and lipids. The tissue residues were then divided into two portions; one portion was heated with trichloroacetic acid as described by Schneider (1945a), the other was treated with alkali as described by Schmidt and Thannhauser (1945). In addition, after DNA and PNA had been separated in the latter method, the DNA was brought into solution by heating the protein-DNA precipitate with trichloroacetic acid. DNA and PNA were then estimated in both procedures by the diphenylamine and orcinol reactions as well as by appropriate phosphorus determinations. The results (Schneider, 1946a) showed that the PNA and DNA found in the trichloroacetic acid extracts by the colorimetric reactions used by Schneider (1945) were, in general, somewhat lower than the PNA and DNA calculated from the phosphorus determinations used in the method by Schmidt and Thannhauser (1945). On the other hand, the amounts of PNA and DNA that were found by the colorimetric reactions of the pentoses in the two procedures were in excellent agreement. Thus it can be concluded that although the method of Schneider (1945a) and the method of Schmidt and Thannhauser (1945) yielded essentially the same results, the agreement which was obtained between the two methods was considerably enhanced if the nucleic acids were estimated by the colorimetric reactions of the pentoses present in the nucleic acids rather than by means of phosphorus determinations.

Recently, Euler and Hahn (1946) described a new method for the determination of PNA in tissues. In this method, the tissue was extracted *directly* with 10% NaCl followed by 0.2 N NaOH. The extract was treated with lanthanum to precipitate the nucleic acids as lanthanum salts. After removal of the lanthanum, PNA was determined by a new colorimetric reaction which employed phloroglucinol. Added PNA was quantitatively recovered and evidence was presented to show that the extraction was quantitative. No evidence was presented, however, that the PNA extracted from the tissue was completely precipitated by lanthanum. Furthermore, the possibility that other pentose-containing compounds were extracted and precipitated by lanthanum acetate was apparently not considered. The results obtained by Euler and Hahn are essentially in agreement with those of other workers. Euler and Hahn did, however, find much higher concentrations of PNA in skeletal and cardiac muscle than did Schneider and Klug (1946). It would appear, therefore, that a careful comparison of the methods of Schneider (1945a), Schmidt and Thannhauser (1945) and Euler and Hahn (1946) on the same sample of tissue would be desirable. Such a study should, of course, include measurements of phosphorus, nitrogen, pentoses, and nitrogenous bases in order that a conclusion might be reached as to whether nucleic acids were actually being measured in all three methods or whether other pentose-containing compounds might be present.

In summary it may be stated that regardless of the method which is chosen for the analysis of nucleic acids, it cannot be emphasized too strongly that the method be checked for completeness of extraction and that the analytical values obtained for each nucleic acid be checked by independent determinations of the phosphorus, the nitrogenous bases, and the pentoses. Agreement between such independent determinations is convincing proof for the validity of the method. Since such proof has been presented for the methods of Schneider (1945a) and Schmidt and Thannhauser (1945) (Schneider, 1946a), it would appear that these methods are preferable until other methods can be checked in a similar manner.

The trichloroacetic acid method of Schneider (1945a) has been applied in studies of the nucleic acid content of normal and neoplastic tissues, the intracellular distribution of nucleic acids, and the relation between pentose nucleic acid and respiratory enzymes. These studies will be described in the subsequent paragraphs.

The Nucleic Acid Content of Tissues

A number of normal and cancerous rat and mouse tissues have been analyzed for nucleic acids, using the trichloroacetic acid method (Schneider, 1945a). The results are summarized in Table 1. The effectiveness of the method is best tested by comparing the ratio of the total phosphorus found in the

nucleic acid extract to the nucleic acid phosphorus calculated to be present from the results of the diphenylamine and orcinol reactions. These ratios are given in the last column of Table 1. It is evident that for most of the tissues studied the ratio closely approached the theoretical value of 1.00.

A comparison of the PNA and DNA contents of the normal tissues studied indicates that the amounts of these nucleic acids varied considerably in the different tissues. This was in marked contrast to the tumor tissues, in which the PNA and the DNA contents were relatively constant. These findings are reminiscent of enzyme studies (DuBois and Potter, 1942; Greenstein, 1945; Schneider and Potter, 1943b) in which the enzyme activity varied considerably in the different normal tissues while the activity of different tumors was relatively constant. Furthermore, since the enzymatic activity of hepatomas resembled that of embryonic liver more closely than that of adult liver (Greenstein, 1945; Potter, Schneider and Liebl, 1945), Greenstein (1945) suggested that cancer tissues probably represent a reversion to a more primitive type of metabolism. The nucleic acid data would appear to indicate that cancer tissue may be primitive in ways other than enzymatic pattern.

TABLE 1. THE NUCLEIC ACID CONTENT OF NORMAL AND MALIGNANT TISSUES

(Schneider, 1945b, and Schneider and Klug, 1946)

	No. of analyses	PNA* phosphorus	DNA* phosphorus	$\frac{P_{DNA}+P_{PNA}}{P_{found}}$
Rat skeletal muscle	11	6.7	5.7	0.79
Rat cardiac muscle	12	12.4	14.5	0.81
Rat brain	3	17.5	12.3	1.01
Rat lung	9	18.0	60.5	1.00
Rat kidney	6	27.2	37.9	0.96
Rat thymus	8	37.8	264.0	1.01
Rat spleen	5	42.5	129.0	1.14
Rat liver	6	63.4	25.4	0.98
Rat pancreas	10	179.0	45.2	0.90
Rat liver tumors	9	54.1	66.7	0.99
Flexner Jobling rat carcinoma	9	49.6	56.9	0.93
Jensen rat sarcoma	7	53.2	66.3	0.95
Walker No. 256 rat carcinosarcoma	5	59.5	66.3	0.96
Mouse lung	8	22.6	58.0	1.18
Mouse lung tumor	7	59.6	104.0	1.03
Mouse mammary tumor	6	56.3	75.0	0.96
Mouse ear tumor (U.V.)	5	76.0	93.6	1.12

* Mg. per 100 gm. fresh tissue; the phosphorus was calculated from pentose measurement.

A comparison of interest is that of homologous normal and cancer tissues. Two sets of such tissues were analyzed for nucleic acids; rat liver and rat liver tumor, mouse lung and mouse lung tumor. The results (Table 1) indicated that the DNA content of the tumors was much higher than that of the normal tissues. The PNA content of the lung tumor was also much higher than that of normal lung, while the PNA content of the liver tumor was somewhat lower than that of the normal liver.

Caspersson and his associates (1939, 1940, 1941) and Brachet (1945) have found independently that the cytoplasm of cells which are actively dividing or engaged in protein synthesis contain high concentrations of material with the properties of nucleic acid and have suggested that nucleic acids may be important in protein synthesis. Our own results (Table 1) show that PNA (the cytoplasmic nucleic acid) is present in high concentrations in the tumor tissues and in the pancreas and liver. Thus, the results confirm those of Caspersson and Brachet. Although high concentrations of PNA appear to be present in tissues actively synthesizing protein, it does not necessarily follow that PNA is involved in protein synthesis. Nevertheless some evidence has recently been presented by Spiegelman and Kamen (1946) to indicate that nucleic acid may be involved directly in protein synthesis. These authors observed that turnover of nucleoprotein phosphorus occurred only during enzyme synthesis and suggested that nucleoprotein phosphorus served as a source of high energy phosphate. No differentiation was made as to whether pentose or desoxypentose nucleoproteins were involved. More recently Novikoff and Potter (1947) have studied the nucleic acid content of rat liver after partial hepatectomy. These authors used the trichoroacetic acid method of nucleic acid extraction (Schneider, 1945a) and have generously permitted the description of some of their results. The data (Fig. 1) show that the PNA content per mg. of dry liver increased rapidly after partial hepatectomy and reached a maximum 2 days after the operation. The PNA content then rapidly declined to normal levels. The weight of the liver, however, continued to increase for some time after the PNA content had reached a maximum level. The results suggest that the PNA (or pentosenucleoprotein) synthesized during the early stages of liver regeneration may be necessary for the synthesis of other compounds. Such an explanation would account for the decline in the PNA content of the liver as a dilution of PNA proteins during the production of other compounds. Since Brues, Tracy, and Cohn (1944) observed a more rapid turnover of PNA phosphorus in regenerating liver than in normal liver, it would appear to be well worthwhile to reinvestigate the turnover of PNA phosphorus at various stages after partial hepatectomy. Such studies are also indicated because of the new methods for the extraction and estimation of the nucleic acids which have become available since the results of Brues, Tracy and Cohn were reported.

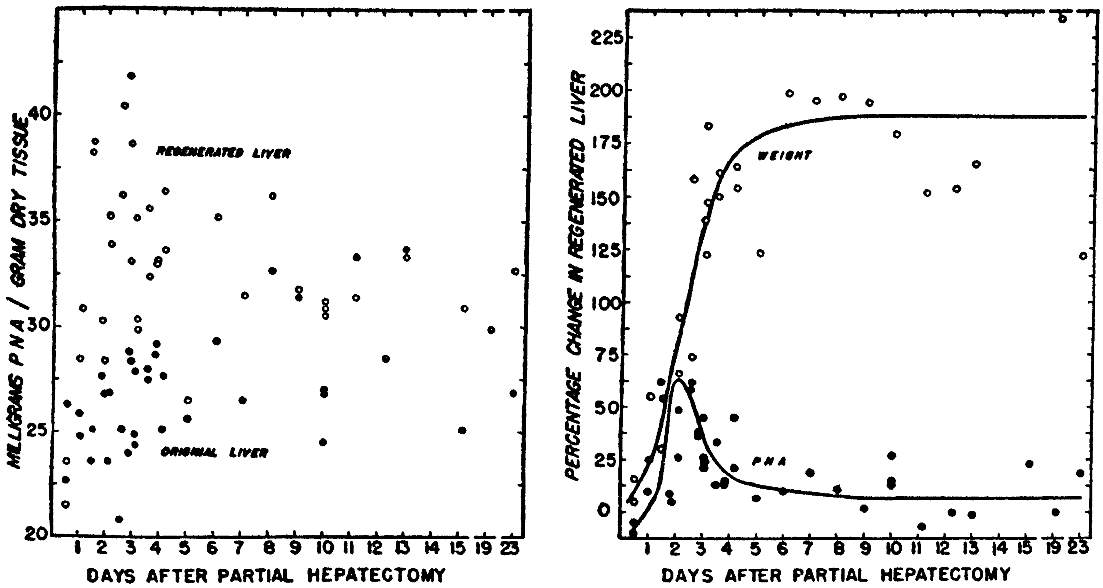

FIG. 1. The pentose nucleic acid (PNA) content of rat liver at various stages after partial hepatectomy (Novikoff and Potter, 1947).

INTRACELLULAR DISTRIBUTION OF NUCLEIC ACIDS

The distribution of nucleic acids within the cell has also been studied in a few tissues. In these studies the cells were disrupted in the all-glass apparatus of Potter and Elvehjem (1936) and were fractionated in the centrifuge essentially as described by Claude (1941, 1944). Rat liver, rat kidney and rat liver tumor cells were disrupted and separated into a nuclear fraction, a large granule fraction, and an unfractionated residue (Schneider, 1946c, d). Microscopical examination showed that the nuclear fraction consisted largely of nuclei which had clumped together, some unbroken cells and some large granules. The large granule fraction consisted entirely of spherical bodies which were about 0.5 to 2.0 μ in diameter. The large granules were most easily seen in the dark-field microscope. Some large granules were also seen in the unfractionated residue. The results of the nucleic acid analyses are summarized in Table 2. The data are expressed as micrograms of nucleic acid phosphorus per mg. of dry material and as per cent of the nucleic acid phosphorus present in the original tissue homogenate. Thus the first method of expression of the

TABLE 2. INTRACELLULAR DISTRIBUTION OF NUCLEIC ACIDS

(Derived from Schneider, 1946c, d)

Tissue	Tissue fraction	PNA phosphorus*		DNA phosphorus*	
		Per mg. dry weight	% of homogenate	Per mg. dry weight	% of homogenate
Rat kidney	Homogenate	1.27	(100)	1.50	(100)
	Nuclei	0.83	10.7	9.13	100
	Large granules	1.00	13.2		
	Unfractionated residue	1.35	74.9		
Rat liver	Homogenate	2.04	(100)	0.71	(100)
	Nuclei	1.49	7.5	7.10	103
	Large granules	2.00	17.5		
	Unfractionated residue	2.05	73.4		
Rat liver tumors	Homogenate	2.92	(100)	2.46	100
	Nuclei	1.40	11.9	9.44	95.8
	Large granules	3.88	10.6		
	Unfractionated residue	3.64	80.6		

* The phosphorus was calculated from pentose measurements.

FIG. 2. A photograph of a smear of a rat liver homogenate (in 30% sucrose) which was fixed with osmium tetroxide and stained with aniline-acid fuchsin-picric acid. Note the liver cell (large, dark mass lacking detail) surrounded by a large number of free mitochondria seen as rods, rodlets, and granules. Magnification × 1125. Enlarged × 1.4.

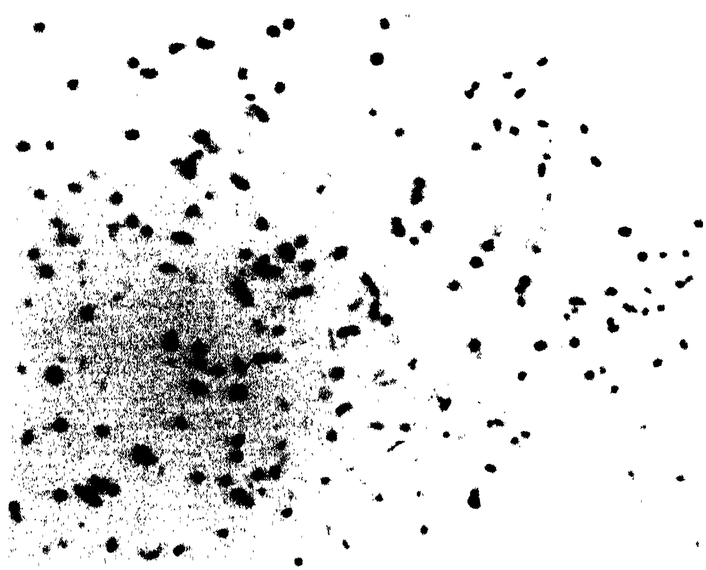

FIG. 3. A photograph of a smear of mitochondria which were isolated from a homogenate of rat liver in 30% sucrose and were fixed with osmium tetroxide and stained with aniline-acid fuchsin-picric acid. Note that the mitochondria are indistinguishable from those in Fig. 2. Note also a few poorly defined spherical bodies (ghosts). Magnification × 1125. Enlarged × 1.4.

results is a measure of the concentration of nucleic acid in a given tissue fraction while the latter method indicates the proportion of the total tissue nucleic acid present in a particular tissue fraction. The effectiveness of the trichloroacetic acid method of nucleic acid extraction was indicated by the fact that the entire PNA and DNA present in the tissue homogenates was recovered in the three tissue fractions.

The results of the PNA analyses (Table 2) show that this nucleic acid was less concentrated in the nuclear fraction of the three tissues studied than in the original tissues. Whether the PNA present in the nuclear fraction was actually present in the nuclei is difficult to decide, since this fraction was contaminated by large granules. On the other hand it would appear improbable that the large granule contamination could account for the entire PNA found in the nuclear fraction. An estimate of the amount of the large granule contamination can be obtained by measurement of the succinoxidase activity of the nuclear fraction since this enzyme system appears to be associated exclusively with the large granules (see next section). Such estimates indicate that large granule contamination would account for less than 20% of the PNA found in the nuclear fractions obtained from the rat kidney, liver, and liver tumors. Thus the nuclei would appear to contain appreciable amounts of PNA. Although PNA was also found in the large granule fractions, the only fraction in which significant concentration of the PNA was obtained was the large granule fraction of the liver tumor. The largest amounts of PNA were found in the unfractionated residues of the three tissues studied perhaps in association with the microsomes of Claude (1941, 1944) or in the form of nucleoproteins or nucleic acids of relatively low molecular weight.

The DNA analyses showed that all of this nucleic acid was recovered in the nuclear fraction. The chemical data thus afford excellent confirmation for cytological evidence which has indicated that this nucleic acid is found only in the nucleus of the cell. It is rather interesting to note that although the DNA content of the three tissues was markedly different, the DNA content of the nuclear fractions was almost the same. In the case of the liver and the liver tumor this would be interpreted to mean either that the tumor had nuclei of a larger size than the liver or a larger number of nuclei per volume of tissue. Since Biesele (1944) has found that the two tissues have the same size distribution of nuclear volumes, the latter explanation would appear to be the correct one.

TABLE 3. INTRACELLULAR DISTRIBUTION OF ENZYMES

(Derived from Schneider, 1946c, d)

Tissue	Tissue fraction	Succinic dehydrogenase		Cytochrome oxidase		ATP-ase	
		Specific activity*	% of homogenate	Specific activity*	% of homogenate	Specific activity**	% of homogenate
Rat kidney	Homogenate	154.0	(100)				
	Nuclei	72.0	7.6				
	Large granules	639.0	69.1				
	Unfractionated residue	43.0	19.5				
Rat liver	Homogenate	72.0	(100)	197.0	(100)	108	(100)
	Nuclei	46.2	6.6	100.0	5.4	280	26.8
	Large granules	304.0	75.5	786.0	74.0	292	48.1
	Unfractionated residue	11.7	11.9	37.8	14.6	44	29.7
Rat liver tumors	Homogenate	24.4	(100)	90.6	(100)	150	(100)
	Nuclei	11.3	11.5	33.3	9.1	75	12.4
	Large granules	171.0	55.9	717.0	63.0	230	12.2
	Unfractionated residue	8.9	23.5	43.9	31.4	172	74.5

* Activity expressed as Q_{O_2} cu. mm. O_2 absorbed per mg. dry weight per hour.

** Activity expressed as micrograms phosphorus liberated from ATP per mg. dry weight per hour.

RELATIONS BETWEEN NUCLEIC ACIDS AND ENZYMES

The rat liver, rat kidney, and rat liver tumor fractions which had been analyzed for nucleic acids (Table 2) were also assayed for enzyme activity. Succinic dehydrogenase and cytochrome oxidase activities were determined by the method of Schneider and Potter (1943a) and adenosinetriphosphatase (ATP-ase) activity was determined by the method of DuBois and Potter (1943). The results are summarized in Table 3 and are expressed as specific enzyme activity and in terms of the per cent of the total enzyme activity of the whole tissue

homogenate which was associated with each fraction. It is to be noted that in each case, almost all of the enzyme activity present in the original tissue was recovered in the tissue fractions. Thus the validity of the enzyme assays was assured.

The data (Table 3) clearly show that in the tissues studied, the activity of the enzymes succinic dehydrogenase and cytochrome oxidase was concentrated in the large granule fractions. The activity associated with the large granule fractions also accounted for a large percentage of the activity present in the original homogenates. Since the enzyme activity associated with the nuclear fraction and with the unfractionated residue could be explained by incomplete separation of large granules from these fractions, it can be concluded that in the intact cells these enzymes are probably localized in the large granules. The study of the distribution of ATP-ase activity showed that in the liver the enzyme activity was concentrated in both the nuclear and in the large granule fractions, while in the liver tumors the activity was concentrated in the large granule fraction and in the unfractionated residue. Since the specific activity of the three enzymes in the large granule fraction of the liver tumor was almost the same as the specific activity in the large granule fraction of the liver, the conclusion was reached that in the transformation of the liver cell into the tumor cell, a considerable amount of large granule material with its attendant enzymatic activity must have been lost. The malignant change cannot, however, be described merely as a loss of large granule material from the cell because, although the succinic dehydrogenase and cytochrome oxidase activities of the liver tumor were 33-50% as great as the activity of the liver, the ATP-ase activity of the tumor was 50% higher than that of the liver but the distribution of ATP-ase activity was profoundly different in the liver tumor than in the liver. Thus the change from the normal to the malignant cell may involve loss of some enzymes and the selective retention of others. Further evidence that the malignant change could not be described completely as a loss of large granule material from the normal cell was supplied by the fact that the PNA content of the liver tumor large granules was almost twice as great as that of the liver large granules. From these results it can be appreciated that the neoplastic change is far from simple. A clearer understanding of the mechanism of carcinogenesis will undoubtedly be obtained as these studies are extended to include the intermediate stages in the carcinogenic process as well as a study of carcinogenesis in other tissues.

The experiments of Potter and Albaum (1943) indicated that PNA may be necessary for respiratory enzymes. These authors had observed that when rat liver homogenates were incubated with crystalline ribonuclease, complete inhibition of succinoxidase and cytochrome ovidase activity resulted. This observation was confirmed (Schneider, 1946b). In view of the fact that these enzymes and PNA were found to be present in the large granule fraction of lymphosarcoma (Claude, 1944) and of other tissues (Tables 2 and 3), it was considered of interest to test the effect of ribonuclease on large granules. Accordingly, the large granules were isolated from rat liver (Schneider, 1946b) and incubated with ribonuclease for varying periods of time. It was found that 85% of the PNA present in the large granules was liberated in 2.5 minutes, while the succinoxidase activity was decreased only 35% after 100 minutes of incubation. Thus, the inhibition of this enzyme system by ribonuclease was apparently unrelated to release of PNA and was only slight in the large granule fraction. Although complete inhibition of succinoxidase activity was obtained in the homogenate, an explanation for the differences in the behavior of ribonuclease toward the homogenate and the large granule fraction was not sought because subsequent experiments showed that two other crystalline preparations of ribonuclease produced only slight inhibition of succinic dehydrogenase. Thus the inhibition which had been observed was apparently due to an impurity in the crystalline ribonuclease sample (possibly a proteolytic enzyme, Schneider, 1946b). Although these experiments suggested that PNA and succinoxidase activity were unrelated, the results were inconclusive inasmuch as it was never possible to remove all of the PNA from the large granules with ribonuclease. Thus the possibility remained that the entire succinoxidase activity was associated with the PNA which was not liberated by ribonuclease. Recently Hogeboom (1946) was able to bring a portion of the succinic dehydrogenase activity associated with the liver large granules into solution. In collaboration with Dr. Hogeboom (unpublished experiments) it was found that these solutions also contained PNA. Fractionation of the solutions with ammonium sulfate resulted in a considerable, but incomplete separation of PNA and succinic dehydrogenase. Final proof as to whether succinic dehydrogenase is a pentose nucleoprotein or requires PNA for its activity will probably await the complete purification of the enzyme.

The Isolation and the Biochemical Properties of Morphologically Intact Liver Mitochondria

The experiments described in the preceding paragraphs have dealt with the large granules isolated from rat kidney, rat liver, and rat liver tumors. It has been suggested (Claude, 1944; Hogeboom, Claude, and Hotchkiss 1946) that the large granules probably represent the mitochondria of the cell since both the large granules and the mitochondria had approximately the same size. Recently, the isolation of morphologically intact rat liver mitochondria was accomplished (Hogeboom, Schneider

and Pallade, 1947). It was observed that when rat liver cells were disrupted in a solution of sucrose in distilled water (30 g. sucrose in 100 ml. solution), a large number of rods, rodlets, and granules which could not be distinguished morphologically from the mitochondria seen inside the liver cell were visible under the microscope (Fig. 2). The identification of these bodies as mitochondria was strongly supported by the fact that they could be stained with Janus Green B at dye concentrations as low as 1:20,000 and 1:40,000.

The isolation of the mitochondria was accomplished as follows: a 10% homogenate of rat liver (Potter and Elvehjem, 1936) in 30% sucrose was centrifuged 3 times for periods of 10 minutes at 600 × g to remove nuclei, red cells, and unbroken liver cells. The mitochondria were then sedimented by centrifuging the nuclei and cell-free extract for 20 minutes at 24,000 × g. The mitochondria were washed once by suspending them in 30% sucrose and centrifuging at the same speed.

The washed mitochondria were resuspended in 30% sucrose and studied cytologically and biochemically. The suspension was seen to consist of a large number of rods, rodlets, and granules all of which were stained at low concentrations of Janus Green B (1:20,000 to 1:40,000). None of the mitochondria were agglutinated. A photograph of a suspension of washed mitochondria which had been fixed with osmium tetroxide and stained with aniline-acid fuchsin-picric acid is presented in Fig. 3. The appearance of the mitochondria is similar to the appearance of the mitochondria in the original liver homogenate (Fig. 2). However, in the photograph of the washed mitochondria, a number of indistinct spherical bodies (ghosts) are evident. The number of the latter is very small in fresh preparations of mitochondria but is markedly increased by fixation with osmium tetroxide. The rapid formation of ghosts was also noted when thin films of the mitochondria suspension were observed under the microscope. The transformation of the mitochondria into spheres and ghosts was noticed when the mitochondria suspension was diluted with 2 volumes of water. Large numbers of spheres were also seen when rat liver cells were disrupted directly in 8.5% sucrose. No rods or rodlets were evident at this sucrose concentration. These observations would appear to support the hypothesis that the large granules are mitochondria or are derived from mitochondria.

Preliminary studies of the physical and biochemical properties of the isolated mitochondria have been made. When the mitochondria suspension was stirred, pronounced birefringence of flow was noticed. It was concluded that this property of the mitochondria suspension was due to the rod-like shape of the mitochondria which had been observed under the microscope. The mitochondria suspension has also been analyzed for nucleic acids and assayed for succinoxidase activity. The results of these determinations are presented in Table 4. The data show that the mitochondria suspension contained PNA but that the concentration of PNA in the mitochondria was lower than the PNA concentration in the original liver homogenate. The data also show that essentially all of the succinoxidase activity associated with the nuclei and cell free extract of the liver was recovered in the washed mitochondria suspension. Surprisingly enough the data on the PNA concentration and the succinoxidase activity of the mitochondria were similar to those which had been obtained with the large granules isolated from water homogenates of rat liver (Tables 2 and 3) or from saline extracts of rat liver (Hogeboom, Claude and Hotchkiss, 1946). Thus the biochemical data constitute further evidence that the large granules and the mitochondria are homologous. Although the isolation of morphologically intact mitochondria has yielded biochemical data which was similar to that obtained with isolated large granules, it may be that in the study of the more sensitive

TABLE 4. THE NUCLEIC ACID CONTENT AND THE SUCCINOXIDASE ACTIVITY OF THE MITOCHONDRIA AND OTHER FRACTIONS ISOLATED FROM RAT LIVER

Rat liver fraction	PNA Phosphorus*		DNA Phosphorus*		Succinoxidase Activity	
	Per mg. nitrogen	Per cent of homogenate	Per mg. nitrogen	Per cent of homogenate	Specific activity**	Per cent of homogenate
Homogenate	23.4	(100)	9.0	(100)	650	(100)
Nuclear	22.1	24.8	36.5	107	511	20
Mitochondria (washed)	13.1	12.5			2008	69
Washing from mitochondria	41.5	11.0			300	3
"Microsomes" (washed)	52.5	17.3			200	3
Washing from "microsomes"	34.0	5.4				
Supernatant fluid	15.4	20.2				

* The phosphorus was calculated from pentose measurements.

** Expressed as cu. mm. O_2 absorbed per hour per milligram nitrogen.

biochemical reactions, the isolation of morphologically intact mitochondria will prove to afford a considerable advantage.

Particles similar to the microsomes of Claude (1941, 1944) have also been isolated from liver cells disrupted in 30% sucrose. This was done by centrifuging the supernatant which remained after nuclei, unbroken cells, and mitochondria had been removed from the liver homogenate. After centrifuging for 2 hours at 32,000 × g, a perfectly transparent red pellet resulted. The pellet was resuspended in 30% sucrose and recentrifuged at the same speed. The suspension of washed "microsomes" exhibited pronounced birefringence when the suspension was stirred. Thus the washed "microsomes" would appear to consist at least in part of asymmetric particles. Analysis of the washed "microsomes" disclosed the fact that these particles contained a high concentration of PNA. (The high concentration of PNA in the washings from the mitochondria and the "microsomes" (Table 4) can perhaps be explained by the presence of "microsomes"). Although the "microsomes" represent the first fraction of the liver in which a concentration of PNA was observed, the significance of this observation can not be assessed at the present time. It will remain for future research to determine what functions the "microsomes" possess and whether PNA is necessary for these functions.

Summary

The results reported in this paper may be summarized briefly as follows:

1. The methods available for the estimation of the nucleic acids in animal tissues were described and discussed.
2. The nucleic acid content of a large number of normal and malignant rat and mouse tissues was determined. It was noted that the nucleic acid content of the tumors analyzed was very nearly the same while the nucleic acid content of the different normal tissues varied greatly.
3. Rat liver, rat liver tumor, and rat kidney cells were disrupted and fractionated with the centrifuge into a nuclear fraction, a large granule fraction, and an unfractionated residue. The nucleic acid contents and enzyme activities of the different fractions were determined. The entire desoxypentose nucleic acid of the three tissues was recovered in the nuclear fraction. Pentose nucleic acid was found in all three fractions but the major portion of this nucleic acid was present in the unfractionated residues. Succinoxidase and cytochrome oxidase activities were found to be associated almost exclusively with the large granule fractions. Adenosinetriphosphatase activity was associated with the nuclear and large granule fractions of the liver and with the unfractionated residue of the liver tumor.
4. Although some evidence was obtained to show that the pentose nucleic acid present in the large granules was not necessary for succinoxidase activity, no conclusive evidence could be obtained that the two were unrelated.
5. The isolation of morphologically intact mitochondria from rat liver was described. The mitochondria were observed to have a rod-like shape and were stained vitally with Janus Green B. Pentose nucleic acid and succinoxidase activity was found to be associated with the mitochondria. Other bodies which appeared to be rod-like and were much smaller than the mitochondria were also isolated from rat liver.

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DISCUSSION

STOWELL: I believe that additional evidence would be desirable on Dr. Schneider's suggestion that the nucleic acid content of tumors, like that of most enzymes, is intermediate in amount as compared with normal tissues. He has presented results on the nucleic acid content of lung and liver tumors and their respective normal tissues. In attempting to make such comparisons, I feel that it is important to relate the results on the tumor cells to comparable data on the normal cells from which such tumors arise. With some tumors this may be especially difficult to accomplish by the usual chemical methods. For example, most carcinomas of the lung arise from bronchial epithelium. It would of course be difficult to interpret the comparison of a chemical analysis of whole lung containing only a few percent of bronchial epithelial cells with a lung tumor which contains other constituents besides neoplastic cells. Likewise, it is important to use a uniform type of liver tumor which does not arise from bile ducts, if comparisons are to be made with normal liver parenchymal cells. In my experience, hepatomas induced by p-dimethylaminoazobenzene differ greatly in their cellular morphology, composition and suitability for analysis. Volumetric measurements of some of my liver tumors, which were free of large areas of necrosis, showed great variation with in some instances as little as 10% neoplastic cells and as much as 60% connective and vascular tissue, as well as appreciable amounts of tissue spaces, necrosis and intermingled non-neoplastic liver cells. Such important factors could readily explain some of the variable results recorded in the literature. I mention such difficulties in the chemical analysis of tumors because of their general importance and I do not mean to imply that they are especially applicable to Dr. Schneider's work, since I think that he is probably more cognizant of these factors than many other chemists.

In recent years I have had occasion to compare the nucleic acid content of nearly 40 human and murine tumors with their homologous normal tissues. Measurements of thymonucleic acid were made by carefully controlled photometric determinations of the light absorption of Feulgen stained tissues. In 9 tumors the amount of ribonucleic acid

was estimated photometrically by the use of pyronine and ribonuclease. The results were expressed by comparing the ratios of absorption by the stain for the tumor and respective normal tissues in terms of amount per unit volume of tissue and per cell. The method, which has been described in the literature, has the distinct advantage that it permits the visualization of the exact cells that are being measured and the elimination from the measurements of other types of tissue and artifacts such as connective tissue, vascular tissue and cells, inflammatory cells, areas of necrosis or keratinization. Furthermore, it is possible to apply statistical analysis and to compare the photometric results with quantitative morphologic data such as the size of the cell, nucleus, nucleolus and cytoplasm, which can be very important in the correct interpretation of data. The limitations of such methods have already been discussed at this and other symposia.

As compared with the homologous tissue, I found the amount of thymonucleic acid per unit tissue volume in 31 human tumors was increased in 29 (statistically significant in 21) and the amount per cell was increased in 21 (statistically valid in 14). In no tumor yet examined has the amount of thymonucleic acid been significantly decreased. The ribonucleic acid content was increased in about half the neoplasms measured.

Dr. Schneider has wisely refrained from attempting sweeping conclusions from limited data on tumors. Although we have considerable evidence that many tumors have disturbances in their nucleoproteins, the changes are difficult to interpret with our present scant knowledge. We do not know yet whether there is a characteristic type of disturbance always present as a specific property of neoplastic cells or what alterations occur in other types of pathologic and normal growth. We should ascertain whether there are qualitative or functional changes in the nucleoproteins, whether the changes are produced primarily or as a secondary change of less significance, as well as how they are elicited and the effects produced. Although we know a little about the nucleic acid component of nucleoproteins, we must remember that the proteins, about which we know less, may have an even more important role. In the eventual elucidation of these complex questions we will need to apply many different chemical, histochemical and cytochemical technics and to be cognizant of the advantages and limitations of each method.

SCHNEIDER: I am aware that the study of animal tissues and especially of tumors is open to the criticism that one may not be dealing with a uniform cell population and that in addition to the cells in which one is interested variable amounts of other cells may be included. Although this would appear to be a serious argument against the use of microchemical technics, it does not mean that these methods should be abandoned in favor of methods which can be applied to single cells. Microchemical methods can divulge much information which cannot be obtained with the cytological technics. The data presented in this paper have demonstrated that the nucleic acids of both types apparently have the same composition in a wide variety of normal and tumor tissues. Such data cannot be obtained at the present time by cytological measurements on single cells. It is to be hoped that eventually the microchemical technics will be refined sufficiently to permit such measurements on single cells. In the, meantime it would appear to be wise to continue microchemical studies on as large a number of tissues as possible and eventually to attempt the integration of the results with those which have been obtained by cytological technics.

THE NATURE OF HETEROCHROMATIN

JACK SCHULTZ

The assumption is widely made, and it is of course the simplest, that there must be a constant substance present in all chromosomes to give the gene its local habitation. Yet one may conceive a situation more difficult of analysis, the persistence of patterns of energy transfer, manifested through a variety of substances, present at successive stages of chromosome and gene activity (see for example Astbury, 1941). The distinction between hypotheses of these two types is a basic one; it leads, among other things, to the attempt to find experimental material in which tests of their consequences are possible. This means an attempt to find material in which the genetic activity of particular chromosome regions can be correlated with their chemical nature. The parts of the chromosomes called the heterochromatic regions present such an opportunity. Indeed as will be seen, these chromosome regions provide a case to test whether with the tools at their present disposal geneticists, cytologists and chemists can achieve the correlation they all desire, between the function of a part of a chromosome, that is to say its genetic activity, its chemical composition and its cytological structure.

The Vicissitudes of Heterochromatin

The history of the problem shows the transition from a purely cytological concept to a cytochemical one. The original term was taken from the description of the sex chromosomes as heterochromosomes, [for the earlier literature, see Wilson (1927), for more recent discussion, White (1946), see also Resende (1946)], because they stained differently at meiosis than their autosomal confreres. Somewhat later, in the botanical literature, studies of the "resting muclei" showed masses of material which stained heavily with basic dyes, and were called, in some cases, chromocenters. It was Heitz (1928-1934), in a series of brilliant papers, who produced what may now be termed the classic formulation of the heterochromatin concept. He was able to show that parts of the individual chromosomes differed in the degree to which they changed in the telophase from the compact heavily stained appearance of the metaphase, to the diffusely staining uncoiled interphase. These early studies were based almost exclusively on tissues in which mitosis occurred, and the contrast was always between the differential behavior of the chromosome parts in the successive stages of the mitotic cycle. Those parts which maintained the condensed state, characteristic of the metaphase chromosome, throughout the nuclear cycle, Heitz called the heterochromatic regions.

There was good cytological and genetic basis for considering what the function of these regions might be. In cytology, it was well known that the nuclei of inactive or degenerating cells became condensed, and deeply staining or pycnotic. In the genetic analysis, particularly in Drosophila, it had been shown experimentally that the Y chromosome, the partner of the sex-determining X, had very little effect on the development of the fly, was so to speak almost a "dummy" chromosome (Bridges, 1916). Unit for unit of the metaphase chromosome, the X contained a greater number of genes, and it was in this sense that the idea was advanced that the Y was genetically inactive. Presently, closer analysis of the distribution of the genes in the X chromosome showed that it contained a region, homologous to the Y (or parts of the Y) in which there were also very few genes in a large block of metaphase chromosome (Muller and Painter, 1932). Heitz then extended the analysis to the cytological map of the autosomes. Thus, in Drosophila the correlation was established of the so-called genetically inert regions, and the cytological heterochromatin. The story was a perfectly straightforward one. Later, Muller and Gershenson (1935) suggested that the function of these regions consisted in elaborating accessory substances, which formed the blocks of stainable material. At that time the discussion was purely cytological; it was only later that these regions were discussed in terms of the substances responsible for the staining, the nucleoproteins or the nucleic acids (Schultz, 1936; Caspersson and Schultz, 1938). It seemed a reasonable picture: the heterochromatic regions were "inert" both for genetic and cytological reasons, and what function they had was a nonessential one.

This clear picture became confused however as other types of nuclei were studied, and attempts made to fit them into the frame. In Drosophila for example, the earlier picture came from the neuroblasts (Heitz, 1933; Kaufmann, 1934) and therefore from nuclei in cells that were relatively undifferentiated. When the giant chromosomes of the salivary gland came to be studied, new characteristics of these regions were recognized (Painter, 1933; Bridges, 1935; Prokofyeva-Belgovskaia, 1935). The darkly staining, compact chromocenter of the nerve cell became a mesh-like area, of interconnected strands, some darker than others. Heitz then suggested that there were two types of heterochromatin, α which could not be uncoiled, and β which uncoiled to exhibit the diffuse mesh. These two types of heterochromatin could be distinguished accord-

ing to this account only when the chromosomes grew to their giant size in the salivary glands (Heitz, 1934; Pavan, 1946).

Pavan's interpretation is that the deeply staining blocks of the intermitotic nuclei are not all equivalent; their behavior in the giant chromosomes discloses specific differences in their mode of growth. It should be stated here, parenthetically, that the evidence to date is not conclusive; in Pavan's case particularly, it is not adequate to localize precisely the regions in the giant chromosome responsible for the staining blocks of the intermitotic nuclei. However this may be, the problem is clearly posed: the nuclei in different types of cells, more precisely the chromosomes and the chromosome parts that make them up, have their special characteristics according to the type of cell. On such a basis, there are now two problems to be considered, not one, according to the earlier picture.

According to this earlier picture, the staining blocks were characteristic for a particular group of regions, and hence the study of the function of these regions constituted an analysis of the nature of these staining blocks. But the definition of the heterochromatic regions must be made more strict: they are chromosome regions which have the specific property of remaining as blocks in the intermitotic state. Since these blocks are not an invariable property of the heterochromatic regions, we must differentiate between the function of the regions and the significance of the blocks for chromosome metabolism. Again, the occurrence of blocks of chromatin in the metabolic nucleus does not necessarily indicate heterochromatin sensu strictu. The recognition that each chromosome part has its own nucleoprotein metabolism, that the cycle of mitosis for the chromosome as a whole is the integral of these individual genic contributions, makes it essential to follow the special regions individually in the different types of cell.

There has been, particularly following the work of Darlington and La Cour, a rather unfortunate emphasis on the cyclic change as the identifying characteristic of heterochromatin. These authors have attributed to heterochromatin a lower rate of reaction with the nucleic acids ("regions which fail to maintain the maximum cycle") hence encompassing under the same heading cases in which there are conspicuous staining blocks at interphase, and those in which the reverse occurs at metaphase (a phenomenon which they term nucleic acid starvation). A somewhat similar point of view has been taken by White, on the basis of the difference between the metaphase behavior of the X chromosome and the autosomes of the grasshopper in its spermatogonial divisions. The X stains lightly at metaphase, hence is negatively heteropycnotic, or as Darlington puts it, contains negative heterochromatin. The difficulty that arises, with these as with the other characteristics of heterochromatin that can be enumerated, that put together they come pretty close to including all the possible aspects of chromosome behavior. What results then is the conclusion that there is no specific characterization of heterochromatin, but a "continuous spectrum." The term in this usage is coextensive with the cyclic changes of chromosomes during mitosis. A different point of view is taken here, that the two problems must be treated as separate: we must find out what the properties of the originally defined heterochromatic regions are, and we must find out to what extent these properties are shared by other genes. The central point is the necessity of distinguishing clearly the function of a specific region of the chromosome as it manifests itself in a variety of different tissues.

It is the purpose now, to consider the nature of heterochromatin in Drosophila, where the analysis has progressed far enough to outline the nature of the problem. The chromocentral regions in this animal have been studied in many tissues in an exploratory way, and in a few in great detail. All grades of structural change have been found, from the pycnotic masses of the intermitotic nuclei, to the meshwork already discussed in the giant chromosomes. Of these, the most useful for the present discussion are the conditions in the giant chromosomes which provide a means of detailed structural and cytochemical analysis, and those in the endomitotic nuclei of the nurse cells, in which the behavior of these regions can be followed through a series of intranuclear mitotic cycles (Painter and Reindorp, 1939; Schultz, 1941). We will consider these latter briefly, before discussing the giant chromosomes. The point of critical importance is that the heterochromatic regions in these nuclei can be shown to be reproducing at a slower rate than the other regions of the chromosome. Moreover, the totally heterochromatic Y chromosome, present as a supernumerary chromosome in the females studied, can be seen to have divided perhaps twice when the other chromosomes reduplicated many more times. This observation opens, as pointed out elsewhere, the way for consideration of the relation between gene reproduction and gene function (Schultz 1941, 1944, 1947). For our present discussion, however, it serves to emphasize the danger of identifying a region as heterochromatic solely by its staining reaction in a metabolic nucleus. In the nurse cells the heterochromatic regions are small; in the salivary glands, as we will see, they apparently keep pace with the others, and so on. Much more detailed study is needed, to supply the basis for proper understanding of these diversities in terms of the function of these regions. This done, it may be possible to interpret the cytological pictures of the variable heterochromatin more accurately.

Heterochromatin and Euchromatin in the Salivary Gland Chromosomes

We have already discussed some of the characteristics of heterochromatin in the giant chromosomes in connection with the development of the

heterochromatin concept. It will be desirable to consider the types of structure in the salivary gland chromosomes in relation to their genetic function, then the structure of the heterochromatic regions themselves, before we are equipped to discuss the functional nature of heterochromatin. The types of structure in the salivary gland chromosomes are familiar and have often been discussed by cytologists. It is useful to recall that the bands which constitute the striations of these chromosomes are commonly believed to represent hypertrophied chromomeres (Painter and Griffen, 1937; Metz, 1941), that the nature of this hypertrophy is a matter of speculation, and that the larger the giant chromosome the greater the variety of fine structure there is to be seen. This variety in fine structure depends upon the arrangement of the granules of nucleoprotein in the bands, the thickness of the bands or discs, and upon the association of the bands into units of complex bands, usually doublets. These diverse structures were recognized by the early workers in the field. Those that concern us most at present are the capsules, in which the disc-like nucleoprotein segments form the crust of a pie, with the filling transparent within. The straight bands were believed by Bridges (1938) at one time to consist of pairs of bands; these would then be capsules, as a sort of limiting case. The other variations in the type of band are due chiefly to differences in the degree to which the individual granules composing the band are approximated to each other.

There are in addition differences in the structure of the different regions. Certain of these, characterized usually as far as the structure of their bands is concerned by fine, diffuse banding, form puffs along the cylinder of the chromosome, or bulb-like structures faintly staining. These puff-like regions resemble, as has frequently been pointed out, the nucleoli in their behavior, and will play a part in the present discussion a little later.

The point of major importance is the use of these structural differentiations in evaluating the distribution of the various substances of which the chromosomes are composed. At the level of cytological structure, the differences between the various morphological elements must be related to the substances of which they are composed. Thus the original criterion of heterochromatin when considered in the light of cytochemistry, reduces to the occurrence of large amounts of nucleic acid in specific areas. In the salivary gland chromosomes, the problem is to discover any local chemical differences definitely associated with heterochromatin. This is evidently a part of the larger problem of the analysis of the chemical composition of the chromosomes, of which there has already been considerable discussion in this symposium. It will be well here to recapitulate for the giant chromosomes themselves, the evidence for the distribution of the different substances already identified in chromosomes, in the various structures, and then to return later on to the discussion of the heterochromatic regions.

The heavy bands themselves show a preponderance of nucleic acid of the desoxyribose type, a datum for which the evidence is well known. The absorption spectra of the bands show the maximum of nucleic acid, they stain with basic dyes, give an intense Feulgen reaction, characteristics which, by and large, are removed by the action of desoxyribonucleases. With the pyronin-methyl green stain, the results are variable: the stain is not a clear methyl green, as should be expected wth desoxyribonucleic acid alone. The purplish color, already noted by Brachet (1939) in mammalian tissue, and interpreted by him as evidence of the presence of ribosenucleic acid in the chromosomes, may also, according to a discussion by Kurnick at this symposium, be due to lower polymers of the desoxy-acid. However this may be, more detailed data are needed, both to evaluate the technique and for the study of its application to the salivary gland chromosomes themselves. It is not unlikely that the bands contain some ribose-nucleic acid and, according to the polarization microscope data, lower polymers of the desoxy-acid as well (Caspersson, 1940b). The pyronin-stained inclusions found by Brachet (1944) in the Chironomus salivary gland chromosomes have not been observed in Drosophila.

The characterization of the proteins in the bands is more difficult. The high absorption of the nucleic acid in the ultraviolet masks the lesser protein absorption; special methods, like those developed by Mirsky, Pollister and Ris (this symposium), are needed to use the intrinsic absorption of the bands to define their protein composition. Serra and Queiroz Lopes (1944) have used their arginine test, and found a high concentration of this amino acid in the bands, indicating a basic protein. Similar evidence came with the use of acidic stains (fast green, acid fuchsin, aniline blue, chlorazol black, etc.), all of which were found to stain the bands with different degrees of intensity (Schultz and José, 1944).

The situation in the interband regions (the "interchromomeres") differs considerably from that in the bands. Enzymatic digestion early showed the structure to be dependent on protein (Caspersson, 1936; Mazia and Jaeger, 1939; Mazia, 1941). The absorption spectra show little nucleic acid, and distinct evidence of the absorption maximum of the proteins of the aromatic amino acids: a situation which Caspersson (1941) has interpreted as evidence for the presence of proteins of the "globulin" type. Serra and Queiroz Lopes (1944) found little arginine to be present, consistent with the ultraviolet data. In the same direction, studies of the solubility of acetic acid fixed preparations in buffers at different pH's, with the nucleic acid maintained in a precipitated state due to the addition of lanthanum acetate to the fixative, showed that the interband spaces broke down and went into solution at the more alkaline pH's, higher than 10 (Schultz and José, 1944). The use of acidic dyes, however, shows

a somewhat more complex situation. With fast green, it is apparent that, in the Drosophila giant chromosomes, a "filler" substance, shown by digestion experiments with pepsin to be protein in nature, combines with the dye. But the structural protein does not, remaining glassy and translucent. There are apparently few groups available for combination with the dye. Interestingly enough, after peptic digestion, when acid fuchsin is used as the stain, more dye is taken up by the interband spaces than before digestion, as if there had been new combining groups opened by the digestion. Thus there are two separate types of protein to be considered: one that stains with fast green and is easily soluble in slightly acid solutions; the other non-staining, resistant to acid—a fact first commented on by Marshak (1936) —and highly elastic. It is of course tempting, although still previous, to consider these respectively as the histone and "tryptophane-protein" of Mirsky, Pollister and Ris (1946, and this symposium).

We have then to consider the distribution of these three types of substance in the euchromatic and heterochromatic regions of the salivary gland chromosomes. The major heterochromatic regions of Drosophila, as we have already said, form by association with each other, the chromocentral mass of the salivary gland nuclei. The chromomeres of which this chromocentral mass is composed vary considerably under different circumstances, genetic and environmental. They are usually described as capsules, aggregated in a diffuse mass; a meshwork of bands disaggregated into "chromioles" (Prokofyeva-Belgovskaia, 1935, 1937); the extreme form of this picture is the "diffuse heterochromatin" described by Bauer (1936) in *Drosophila pseudoobscura*. The capsules themselves have a central area that obviously absorbs less ultraviolet light than the interchromomeric spaces, with the encrusted nucleic acid over the edges. There are such capsules found in other regions of the salivary gland chromosomes; they are not, as Bauer (1936a) suggested, diagnostic of heterochromatin. What does seem characteristic is the nature of the interchromomeric space; in the chromocentral regions, when the usual cytological preparations are so made that the chromosomes are stretched, the heterochromatic regions break, between the bands, instead of stretching. The clear difference is then that these regions, with their capsules, do not have the same type of inter-band space as the euchromatic regions. This difference presumably concerns the elastic protein already discussed. It leads us to a closer discussion of the nature of the capsules, the substance within them, and the general structure of the salivary gland chromosomes.

The clue of interest comes from a recent study in our laboratory, of the structure of the salivary gland chromosomes with the phase microscope. It has long been suspected that the capsules are actually fixation artefacts, a view discussed by Metz, for example. In Drosophila it has long been the experience that with slow and gentle fixation (in the cold, for example) the vesiculated or capsular chromosomes are rare. Now, with the study of glands mounted in Ringer's, and observed under the phase microscope, it is clear that in well-handled preparations there are no capsules in the chromosomes. All the bands are closely appressed, clean structures, multiple in some cases and apparently single in others. On fixation, capsule formation can be seen as it occurs under the microscope. The bands that are to form capsules pop open, explode as it were, to form these capsules. And this process occurs for the heterochromatic regions as well as for the others. The question arises, what is inside the capsules, concealed in the living chromosomes? One criterion is provided by the fast green stain, which it will be recalled stains a "filling" substance, with similarities in its behavior to the histones. As I noted some time ago, this stain does not appear within the capsules. In fact, the best interpretation of the microscopic data, and of their mode of origin, is that there is nothing inside the capsules.

Are these then single structures, with a definite plane along which they separate under circumstances of denaturation? Genetic studies by Lewis (1945), of the behavior of one such capsule, supported by less complete evidence in other cases, indicate that the interpretation originally made by Bridges is correct. These capsules appear to be duplicate genes; they are therefore associated with each other at their periphery—although in some cases this varies, the association being at one side only at times ("opened-out figures"). If this is correct, it follows that the fibrous interchromomeric material does not make its appearance between like genes; these are directly associated with each other, nucleoprotein band to band. Such a conclusion would have important repercussions on theories of chromosome structure; they cannot be followed through here in detail. The main question, and one with immediate bearing upon the heterochromatin problem, is what role is to be ascribed to the interchromomeric regions, the interband spaces. As we have seen, they apparently do differ between the major heterochromatic regions and the euchromatin, so-called. On the evidence of the capsules, this fibrous protein is not the continuous material of the chromosomes; it attaches nucleoprotein segment to segment, much as Belling (1933) considered the interchromomeres to grow out from the chromomeres in gene reproduction. But like genes apparently maintain themselves in linear order without any differential intergenic connections, if we make the jump and consider the capsules duplicate genes.

The properties of the heterochromatic regions in the salivary gland chromosomes, from the morphological point of view, are closest to those of a series of duplicate genes. They are "capsules," of similar morphology, connected to each other by strands of

the same nucleoprotein material of which the bands are composed. These regions conspicuously lack the fibrous interchromomeric material. If, arguing from the still meager evidence available, we suppose that the fibrous material only appears between genes sufficiently differentiated with respect to each other to have lost their specific homology, the heterochromatic regions could be groups of duplications of dupiicate genes.

The view that heterochromatin consists of duplicate regions is not a new one. It was first advanced by Prokofyeva (1935), from the consideration of the association of these regions with each other to form a common chromocentral mass. This was interpreted as a synaptic association of homologous regions. The alternative point of view would be that these regions had the property of adhesion or non-homologous association (Schultz, 1939; Schrader, 1941; McClintock, 1933); an association which can only take place where the other member of the associating group likewise can associate non-homologously. In other words, synaptic association may be misleading as a guide to homology and cannot be used as crucial evidence for it.

Another line of argument has been used by Pontecorvo (1944). On the basis that each gene has its own characteristic cycle of nucleoprotein metabolism, he argued that duplications for a gene, tandem along the chromosome, would give rise to regions which would be differentiated from the others, in containing either more or less nucleoprotein than the average. This discussion suffers from most of the confusions encountered in the work on heterochromatin since Darlington and La Cour's attempt at redefinition in terms of cycles. The evidence for blocks of nucleoprotein does not come from the salivary gland chromosomes, but from the intermitotic nuclei. And in these as has already been discussed, it appears that a region amounting to a few bands in the salivary gland chromosomes, is responsible for a large block of the nucleoprotein (Muller, Raffel, Gershenson and Prokofyeva-Belgovskaia, 1937). It is evident that we are not dealing with random duplications of genes, but with a special kind of locus whose properties are to be analyzed. In other words, the special properties of heterochromatin which are to be analyzed, are properties not of the duplications of genes, but of the individual genes themselves. Thus Pontecorvo's argument, while a good one to explain how regions of the chromosome having properties different from the average might arise, has no special relevance to the problem of heterochromatin.

The two older arguments for the homology of the heterochromatic regions fail when critically examined. The present discussion, based on the properties of the interchromomeric regions, can be regarded only as presenting a working hypothesis; more cytogenetical work on the salivary gland chromosomes of Drosophila (*cf.* Kaufmann 1944, 1946), and other suitable organisms is clearly needed. What can be said is that the heterochromatic regions do differ from euchromatic ones in their interchromomeric regions. It should be recalled here that Caspersson (1940b) found differences in the absorption spectra between the regions near heterochromatin and the euchromatic regions proper. These absorption spectra are perhaps best interpreted as due to an as yet unidentified nucleolar material which contains substances absorbing around 2900 A. They can best be discussed in connection with the function of the heterochromatic regions in the over-all metabolism of the nucleus. They seem not to be properties of the heterochromatic regions themselves, but of their products. As has been said, the capsules of the heterochromatic regions in fixed preparations are not too different from the usual capsules in structure. What difference there is consists chiefly in a greater disaggregation of the bands into chromomeric granules. This feature, and the absence of the regular structure of the interband spaces, have been made use of by Ris and Crouse (1945) in a discussion of the structure of the giant chromosomes, which is not irrelevant to the present theme. Their hypothesis is that the giant chromosomes represent uncoiled chromonemata, longitudinally as well as laterally hypertrophied. The differentiation into band and interband regions is due to differential uncoiling, and accumulation of matrical substance in the chromosomes. The bands themselves are believed to be very tightly coiled chromonemata, and the interband regions are uncoiled. The emphasis is upon the continuous chromonema as the fundamental basis of chromosome structure. Heterochromatic regions, on this view, represent regions in which "chromomere" and "interchromomere" have undergone a comparable degree of uncoiling.

The crux of the matter is the structure of the bands, and the mode in which they have grown from the small granular chromomeres visible in the embryonic salivary gland nucleus. There is no theoretical or observational basis for excluding the hypothesis that nucleoprotein structures are spirally wound fibers; and there is good chemical basis for such speculation. This much said, there still is no support for inference regarding the origin of the differentiation by differential uncoiling. What is to be explained is not a process of uncoiling, but a differential synthesis of varied material as the chromosome grows in all dimensions. Since the hypothesis of Ris and Crouse concerns all three substances in the chromosomes, it seems simpler to maintain the distinction between chromomere and interchromomere. And for the heterochromatin problem to which we return, no new insight results from the change of emphasis, from chromomere to differentiated chromonema: at present they are operationally identical. It will be necessary to return to this problem, somewhat later, from the point of view of heterochromatin function.

The Genes Located in Heterochromatin

Thus the cytochemical analysis of the heterochromatic regions as compared with the euchromatin has provided a hypothesis, which has consequences for the genetic function of these regions. If they are duplicate genes, they should have similiar functions, and display allelic properties, it may be said. The difficulty arises, as will be remembered, that the prime pecularity, from the genetic point of view, of the heterochromatic regions, is their apparent lack of the specific type of mutation which lends itself to tests of allelism of the usual type. It was this characteristic that first led to their being called inert regions, and even when definite evidences of activity were found, they turned out to be of a type that made the test ambiguous. It is the problem of the production of non-specific substances which may be produced by all genes, and for which therefore the ordinary test for allelic, homologous genes will not be sufficient. More concretely: in the discussion of the properties of heterochromatin, the problem is to determine how far each property is unique, and how far a joint property with euchromatic regions. For it is conceivable, and has been argued, that these regions are degenerate genes, which have lost the specific qualities that distinguish euchromatic regions (Muller, 1918; see also Muller and Gershenson, 1935; Muller, 1945). This is a concept which implies that the specificities of the genes are separable from their other qualities, a concept which could be justified by considerations of the relations of different types of the nucleoproteins to each other, or even by changes in the type of the nucleic acid. In any case, the problem demands a serious study of the genetic and cytologic functions of heterochromatin, to evaluate the possibilities.

The discussion that follows will be restricted largely to the work on Drosophila, since only here have the genetic potentialities of these regions been studies sufficiently. We will consider first the genes located in these regions, that are known by mutants of the type found in other chromosomal regions. It is of course obvious that any kind of gene might by the accidents of chromosome rearrangement during evolution become transposed to places within the heterochromatic regions. However, there is evidence of functional adjustment to this region for at least three of those located within heterochromatin (Schultz 1939, Panshin 1939). It is therefore significant that one of these genes (bobbed, in the X chromosome) affects the bristles of the fly, another its eye color (light, chromosome 2) and the third the wing pattern (cubitus interruptus, chromosome 4), and that none of these shows any signs of homology with the others. Yet the regions in which they are located, the bands in the giant chromosomes, regularly associate with each other in the chromocenter. Here are genes which still retain specificity, and which also display properties of heterochromatin. On this showing, if the "degenerate gene" hypothesis is to be maintained, the attainment of the heterochromatic state precedes the loss of the specific qualities of the gene. The outlook is even more difficult for a straightforward hypothesis involving duplicate genes; all the cases so far studied still retain some semblance of homology—effects on the same character, etc.—which these genes do not. Thus the consideration of the ordinary genes in heterochromatin shows new complexities of the problem.

Nor has the situation been clarified by Mather's analysis of the effects of the Y chromosome on bristle number in Drosophila melanogaster. His effort is to define heterochromatin as the locus of the "polygenes" which, forming a balanced system, have only slight effects in the aggregate—essentially a system of minor genes, having quantitative effects. Cooper has already made a critique of Mather's experiments, a critique which may be reinforced. It is not surprising that the Y, which itself contains the bobbed gene, affecting the bristles, should affect bristle number. For an attempt to arrive at a general conclusion, applicable to other heterochromatic regions, some other character should have been chosen.

The Genetic Function of Heterochromatin

Let us turn now to the genetic properties of heterochromatin itself. The Y chromosome of Drosophila, as we have previously noted, is "completely" heterochromatic. It was Bridges (1916) who showed that a male without the Y chromosome was sterile, but otherwise completely normal. The genetic map of the Y chromosome is complex. It contains several heterochromatic segments homologous to a part of the X. The genetic details cannot be gone into here (Neuhaus, 1936, 1939), but the question raised is whether the homologous portions of X and Y must be present in two doses for fertility, or not. It turns out that this may be the case: males deficient for the major heterochromatic region of the X, but containing a Y, are fertile (Gershenson, 1933; Sivertzev-Dobzhansky and Dobzhansky, 1933). These could contain the necessary gene in two doses from the regions of the Y which carry it. Sterility ensues however when to the deficient X certain aberrant Y's are added (Schultz, Dubinin and Besmertnaia). For the present discussion, this sterility is of interest as a characteristic of one heterochromatin region. The question is whether evidence of homology with the other heterochromatic regions may be obtained; a question towards whose answer we have at present only some clues of interest. The evidence comes from an analysis of the types of sterility found in rearrangements of the X with other chromosomes.

It has long been noted that, of the X chromosome rearrangements in Drosophilia, those which were viable in the male were frequently sterile. In point of fact, special large scale experiments had to be carried out, to provide a series of rearrangements which were male-fertile. (Patterson, Stone, Bedichek and Suche, 1934). The sterility appears to be inseparable

from the rearrangement, hence is located near the point of breakage, and must be due either to a mutation in genes near the point of break, or, what is more likely, a position effect due to the adjustment of function of genes to their neighbors. The relevance to our present discussion comes from the analysis of the points of breakage. The situation may be summarized in this way: when both breaks are in heterochromatic regions, that is to say, when the X broken in its heterochromatic region is translocated to an autosomal heterochromatic region, the male carrying the rearrangement is usually fertile. When the X is broken in a euchromatic region, and translocated to an autosomal euchromatic region, the result is sterility in the males of this type. For the two complementary classes, involving euchromatin-heterochromatin rearrangements, the situation is somewhat more complex, as will be seen presently, due to changes induced in the euchromatin by proximity to heterochromatin.

From the data at hand, it would appear that there is a relation between the heterochromatin of the different chromosomes, such that they are adjusted to whatever position effects are exerted by each upon its neighbors. This is evidence of similarity of function, of the sort we needed for the characterization of the heterochromatic region as regions having special properties. For the maintenance of fertility in the male, a break in the heterochromatin of the X seems to make little difference. One might argue that there are in the medial region of the X regions especially concerned with fertility; some rearrangements between medial X and autosomal heterochromatin, however, are fertile. These inferences are gathered from data not collected for the purpose; and special experiments are clearly needed. But these are indications that the heterochromatic regions of the different chromosomes do have similar functions.

Clearer evidence comes from the study of the rearrangements between euchromatic regions and heterochromatic regions (Schultz, 1936, 1939; Demerec, 1941; Noujdin, 1935, 1943, 1944). In such rearrangements, the rule seems to be that the euchromatic genes transposed to heterochromatin undergo changes during the histogenesis of the fly. These changes resemble those brought about by mutations of the genes and lead to variegation for whatever characters they may affect. Thus if the genes concerned affect the eye color, the flies carrying the rearrangement show eyes spotted with the mutant color, other genetic conditions being correct: and similarly where other characters are concerned. This is the case, as a general rule, for heterochromatic regions in all the chromosomes. Differences exist between the different regions, and not all parts of the heterochromatin of each chromosome are necessarily concerned in the variegation process. But it is definite, that the occurrence of a specific type of variegation is a genetic characteristic of the rearrangements with heterochromatic regions. The variegation itself is genetically controlled by a complex system of genes, and is most sensitive in its response to the other heterochromatic regions. We will return to the nature of this process in considering the function of heterochromatin in chromosome metabolism.

One further characteristic of the heterochromatic regions is their response to irradiation. Here Muller and his collaborators have adduced evidence for a high breakability of these regions, a criterion which has been extended by Prokofyeva-Belgovskaia (1939) to identify minor heterochromatic regions scattered through the chromosomes. This has been studied by Kaufmann (1939, 1946) who believes that the break frequency is proportional to the mitotic length of the affected areas. On that basis, the high break frequency of the intercalary, smaller heterochromatic regions would be due to their forming at the stage in question, blocks of chromatin (chromomeres) that behave like the major heterochromatic regions. Muller (1945) has more recently attempted a separation of the two variables, by irradiating strains deficient for, and strains containing duplications of the genes producing the intermitotic blocks. The frequency of rearrangement in the two cases was the same, which he interprets as support for the idea that breaks occur between the blocks. The difficulty with the discussion so far seems to be that no distinction is made between an increased breakability due to the relatively larger size of the blocks ("sensitive volume") and one due to a slower rate of regeneration of intergenic bonds after a successful hit. On the latter possibility, the increased frequency of rearrangements would result from a difference in, presumably, the rate of synthesis of fibrous protein between chromomeres, such that new arrangements would have a different chance of being formed. In any case, what is important for our present discussion, so far as the data go, they seem to be characteristic of the heterochromatic regions as a group.

Thus for three characteristics associated with heterochromatin, there is evidence of similar function for the different regions. How far does this contribute evidence that the heterochromatic regions really are duplicate genes? Can such a similarity of function also be interpreted according to the hypothesis of degenerate genes, or specialization in a non-specific function? It is not at all clear at the moment, as has been said, how far these two hypotheses are mutually exclusive; or at least what experimentally testable differences there are between them. Certainly the difference between different genes in heterochromatic regions could be used as argument for the degeneration hypothesis. But if the idea is admissible of the degeneration of specific genes to non-specific ones, there is no reason why there should not exist some genes which have never possessed the specific qualities, that are recognized in the others by the derangements caused by mutation.

Heterochromatin and Chromosome Metabolism

The closer analysis of the nature of the heterochromatin effects, both on fertility, and on the variegation, provides a further step which correlates the cytochemistry and the genetics. First, as regards the effect of the Y chromosome on fertility. Earlier studies, by Safir and by Shen, had indicated that the spermatogenesis of the XO male is normal. This was an error, understandable in view of the methods of staining that these workers employed. What actually happens is that the sperm nuclei do not go through the final stages of the elongation of the sperm head. The head remains in these abnormal males as a round mass, strongly staining in acetic-orcein, and never attains the slender filamentous form of the mature Drosophila sperm. Similar pictures are found in certain of the rearrangements with sterile males; differences of degree are present, the elongation of the sperm head being stopped apparently at different stages. There is in all these cases therefore, an effect upon the behavior of the chromosomes themselves, in their final differentiation in the nucleus of the sperm head. The importance for our present discussion is that these characters associated with heterochromatic regions, are definitely intranuclear, within the individual cell, and have in fact been shown by Stern and Hadorn (1938) to persist in reciprocal transplantation experiments.

The study of the nature of the variegation takes us to the giant chromosomes again, where the attempt has been made to correlate the observed variegation in the adult fly with changes in the chromosomes. The study has been a difficult one, in which there is no complete agreement between different workers in the field, with the literature rather confused. I believe, however, after a careful comparison of my own data with those of such workers as Prokofyeva-Belgovskaia (1939, 1945) that the account presented at the Seventh International Genetics Congress (Schultz, 1939) still stands, and that the discrepancies are only apparent ones. The essential fact is that the regions containing the genes for which variegation is displayed show regular changes that are correlated in their frequency and extent with the frequency and extent of the variegation. The nature of the changes is of more interest for the present discussion than their relation to the variegation.

The extent of the variegation (a quantity determined by the time in development at which it occurs, and the frequency of its occurrence) is easily modified by environmental and genetic factors. Chief among the latter are the other heterochromatic regions; particularly striking are the effects produced by a supernumerary Y chromosome, under whose influence a degree of suppression, in some cases almost a return to the normal, may be observed. With such material, it is possible to follow the various stages in the variegation process.

The process has been described as a "heterochromatization" of the bands in question. Since heterochromatin itself can vary from the diffuse to the densely pycnotic type, it is not surprising that all these stages are seen. Consider a faint band, transposed next to heterochromatin: in individuals showing slight variegation, the band stains more heavily with the Feulgen stain, and as Caspersson and Schultz (1938) were able to show, actually contains more material absorbing where nucleic acid does. This has been disputed by Cole and Sutton (1941), on the ground that their measurements of absorption spectra showed high variability within a single band. As was pointed out in the discussion to their paper in the Symposium on Genes and Chromosomes (p. 70), the technique which they used is valid only for qualitative study. In the work of Caspersson and Schultz, measurements were made at eight points along the band, and the total extinction coefficients for the area of the band obtained in detail. Otherwise a quantitative comparison could not have been made, for the same reasons that caused Cole and Sutton to conclude that no differences exist. Unless the whole bands are compared, the local differences between points within a band can mask whatever differences exist between bands. With the measurements properly made, the maximum increment in nucleic acid appears at the band closest to point of rearrangement, with the effect lessened as distance increases. This parallels the effect on the variegation.

In these slighter cases the integrity of the bands is still maintained. Raised at a lower temperature, where the variegation is more extreme, the bands show the disorganization, the meshwork of attached nucleoprotein that is characteristic of the chromocentral regions. It is with such cases that the major part of Prokofyeva's work has been done, and wherever our material is comparable, I believe our stories agree. It is in still more extreme cases, which I have found in certain variegated white-notch stocks with which, so far as I can tell, Prokofyeva has not worked, that our stories diverge: I find that the region in question is not recognizable, and in fact I have not been able to prove satisfactorily that it is present. This is not the place to discuss these problems in detail, however; the present concern is the bearing of these results on the nature of heterochromatin.

The picture in the giant chromosomes may be taken as the amplification, after growth, of a pattern set in the embryonic salivary gland cell. From this it follows that at that early stage euchromatic regions may have had imposed upon them a heterochromatic form. This could be interpreted as evidence for the idea that heterochromatin itself might have originated as a degeneration product, for here is a case in which euchromatic genes "degenerate." Yet there is a distinction to be observed; here the heterochromatization is imposed by heterochromatin, which raises a serious question as to the com-

parability of the evolutionary hypothesis with the developmental inactivation.

Which of the three substances discussed above as making up the salivary gland chromosomes, are involved in this process of heterochromatization? The nucleic acid of the desoxyribose type, certainly;

TABLE 1. EFFECT OF THE Y CHROMOSOME ON THE END SHAPE OF OTHER CHROMOSOMES IN THE SALIVARY GLANDS OF *Drosophila melanogaster*

These data came from three preparations each, of two types of cross. One cross gave comparable XO and XY males, from matings with the giant apricot stock. The other gave comparable XY and XYY males from crosses with the scute-8 apricot stock. In both cases, the glands of the different genetic compositions were present on the same slide, and fixed and stained under as closely comparable conditions as possible. The ends were classified as "nipples," "straight ends" and "fans," in the XO-XY preparations; for the XY-XYY group, only "nipples" and "straight ends" were differentiated, what "fans" there were probably being included under the straight ended group.

Shape		Chromosome end (% of the different types)			
		2L	2R	3L	3R
nipple	XO	6.4	—	—	—
	XY	23.3	19.7	6.8	14.0
	XY	34.2	38.6	44.9	29.6
	XYY	98.7	96.1	97.2	100.0
straight	XO	27.7	52.5	11.4	36.8
	XY	50.0	75.0	62.7	76.0
	XY	65.8	61.4	55.1	70.0
	XYY	1.3	3.9	2.8	—
fan	XO	57.5	47.5	88.6	63.2
	XY	26.7	5.3	30.5	10.0
	XY	*	*	*	*
	XYY	*	*	*	*
Number of each (not %)	XO	43	38	44	49
	XY	60	56	59	50
	XY	79	70	69	81
	XYY	76	77	73	73

* Not classified in this way.

as far as the two types of protein are concerned, and the ribosenucleic acid, more data are needed. The fibrous protein is most probably affected, since the heterochromatic structure appears. Thus the effect of the heterochromatin in this case as in the case of the sperm head, is on the chromosomes themselves and their structural components. Is this a general effect of heterochromatin?

At the outset of the work on variegation, it was of interest to check as a control the behavior of the normal salivary gland chromosomes with different balance of heterochromatin in the nucleus (Schultz, 1936). It seemed not unlikely, that the rearrangements were simply more sensitive indicators of processes going on in the normal structure, to which the wild type was so adjusted that development proceeded normally. Actually in a more recently studied species, *D. pallidipennis*, Dobzhansky (1944) has found variations in the extent of the meshwork structures of the X chromosome under normal conditions. At that early time, it was found that the structure of the salivary gland chromosomes did in fact change as the heterochromatin in the nucleus was varied: with extra Y chromosomes, they became plumper, and stained more intensely; in the XO male, they became flaccid and pale. I have recently repeated these observations, and have found them to be correct. In the recent work, a more sensitive index of the structure of the chromosomes was found to be the shape of the ends. The giant chromosomes may, broadly speaking, have straight ends, fan-shaped ends, or bulbous, nipple like ends (of Hinton, 1945; Kodani, 1947). It turns out that the "poorer" the chromosomes, the higher the frequency of the fan-shaped ends, which occur in the XO male, while the nipples appear chiefly in the XYY male (Table 1). The number of Y chromosomes present in the nucleus exerts a determining effect on the structure of all the chromosomes.

Again, the question: what substances are involved in this change? The study has not yet proceeded far enough to make a proper answer, in quantitative terms. Observationally, however, it is evident that the bands stain lightly in the absence of the normal Y complement: the nucleoprotein components are affected, and not only in amount, but in arrangement as well. For the bands appear more dispersed, and there are evident nucleoprotein staining fibers between them. There is less of the non-staining fibrous protein the chromosome tends more towards a flat ribbonlike shape. And for the fast green staining component, it seems to go along with the others, although more variable in behavior.

Studies with the phase microscope show that these differences are present *in vivo* also. The XO nucleus has a more watery appearance, the nucleolus is not so compact, the chromosomes are less substantial. From all these preliminary observations, the impression is that some common precursor substance is lacking, which has prevented the synthetic system in the chromosomes from working at its normal level. This idea accords well with the thought that the heterochromatic regions function in what may provisionally be termed, for the lack of more precise statement, as the generalized metabolism of the chromosomes. The fact that such individuals are morphologically normal indicates that there are substances in the chromosomes either present in excess for normal genic function, or substances which do not have to do with the specific functions of the individual genes.

THE RELATION OF HETEROCHROMATIN TO NUCLEOLAR SUBSTANCE

Consideration of how such a mechanism operates leads us to a characteristic of heterochromatin not so far discussed—its relation to nucleolus formation.

The well known work of Heitz (1931) showed that the nucleolus was formed at a part of a chromosome, which McClintock (1934) was able to demonstrate in maize was adjacent to and conditioned by heterochromatic regions. This is also true in the salivary gland nuclei, in which the nucleolus has been associated with one of the bands of the heterochromatic regions of the X and the Y (Kaufmann, 1938), but not with the other heterochromatic regions. According to McClintock's account, the nucleolar region serves to organize material normally formed elsewhere in the chromosomes, into the nucleolar structure. In the absence of the nucleolar organizer, nucleoli are formed along the other regions of the chromosomes. In other words, the type of system that has been discussed for the function of the heterochromatic genes seems to fit the nucleolar system. What the heterochromatic regions can do, the other genes can also do, when they are called upon to act in this way.

The role of the Y chromosome in determining the structure of the salivary gland chromosomes seems to accord with this also. For the nipples at the chromosome ends take the same stain as do nucleoli, a fact of considerable interest in the analysis of the heterochromatic nature of these ends, and their relation to the "telomeres" or end genes of Muller (1941). What this means, however, is something more. In Drosophila the region carrying the nucleolar organizer also contains genes which serve in nucleolar synthesis. One may conceive that the same genes are responsible for both functions, that the role of the region as nucleolar organizer is the result of its superior efficiency in carrying out nucleolar synthesis.

The nucleoli contain ribosenucleoproteins which were shown to vary in composition according to the genetic structure of the animal (Schultz, Caspersson and Aquilonius, 1940). This is the cardinal fact which lies at the base of the theory that the heterochromatic regions have to do with cytoplasmic synthesis in general, by way of precursors deriving from the nucleoli. Measurements of the cytoplasm of the salivary gland cells in the different chromosome types have not yet been made, although differences in staining capacity with pyronin and with fast green seem to occur. It is not unexpected that such differences should occur, since it has already been demonstrated that the Y chromosome influences the nucleic acid content of the egg cytoplasm in Drosophila.

We have thus arrived by a devious route at one of the chief topics of this symposium, the relation of the nucleic acids and nucleoproteins to protein synthesis (Caspersson, 1941; Brachet, 1944). The picture that we have is that of a complex system, in which many components play a part. The same region which synthesizes blocks of desoxyribosenucleoprotein in the chromosomes has to do with ribose nucleoprotein synthesis in the nucleolus and cytoplasm, and with the different protein components of the chromosomes. Its role in the variegation process ties it up with gene reproduction, since in variegation the daughter genes differ from each other. And as we have seen, in the nurse cells there is evidence that these genes can have different rates of reproduction. It is clear that the problem of the nature and action of such genes demands a more detailed description. We have effects on protein synthesis, which must be described in definite chemical terms if they are to be correlated with such pictures of specific gene action as those presented by the workers on Neurospora, or with the work on enzyme synthesis in microorganisms.

To do this, it has been necessary to approach the problem by way of the nutritional requirements of the organism, and devise a chemically defined medium for the growth of Drosophila (Schultz, St. Lawrence and Newmeyer, 1946). This has been done, and preliminary results with one of the variegated races, show that the nutritional differences are indeed complex. I mention this here, because one of the results indicates that there is no difference in the nucleic acid requirements of individuals having a supernumerary Y and the normal genotype. This excludes the simple possibility that there is an overall center of nucleic acid metabolism in the heterochromatic regions. As I said, the work is still in its early phases from this point of view. But it should allow a serious attempt at that correlation of chromosome composition and gene function which is one of our goals.

Summary and Conclusions

1. The development of the heterochromatin concept is discussed. The heterochromatic regions are characterized in intermitotic nuclei, as chromosome regions that form massive blocks of Feulgen positive material, *e.g.* desoxyribose-type nucleoprotein. It is shown that two distinct problems have emerged: the role of the genes responsible for the major staining blocks in intermitotic nuclei; and the significance for gene function and metabolism, of the variations in nucleoprotein content of specific chromosome regions at different stages of mitosis, and in different types of nuclei.

2. In the giant chromosomes of Drosophila, where the most detailed analysis has been made, the heterochromatic regions show structural similarities to regions in which duplications of genes have occurred. These similarities are: a) the association of the heterochromatic regions with each other, for which the alternative interpretation of non-homologous association or adhesion is possible; b) the structure of the bands associated with these regions, which form vesicular structures (capsules) on drastic fixation, of a type known in one case to be such a duplication. It is concluded that these similarities are not to be overstressed, and are not the essential characteristics of heterochromatin, although heterochromatic genes like others, may be present

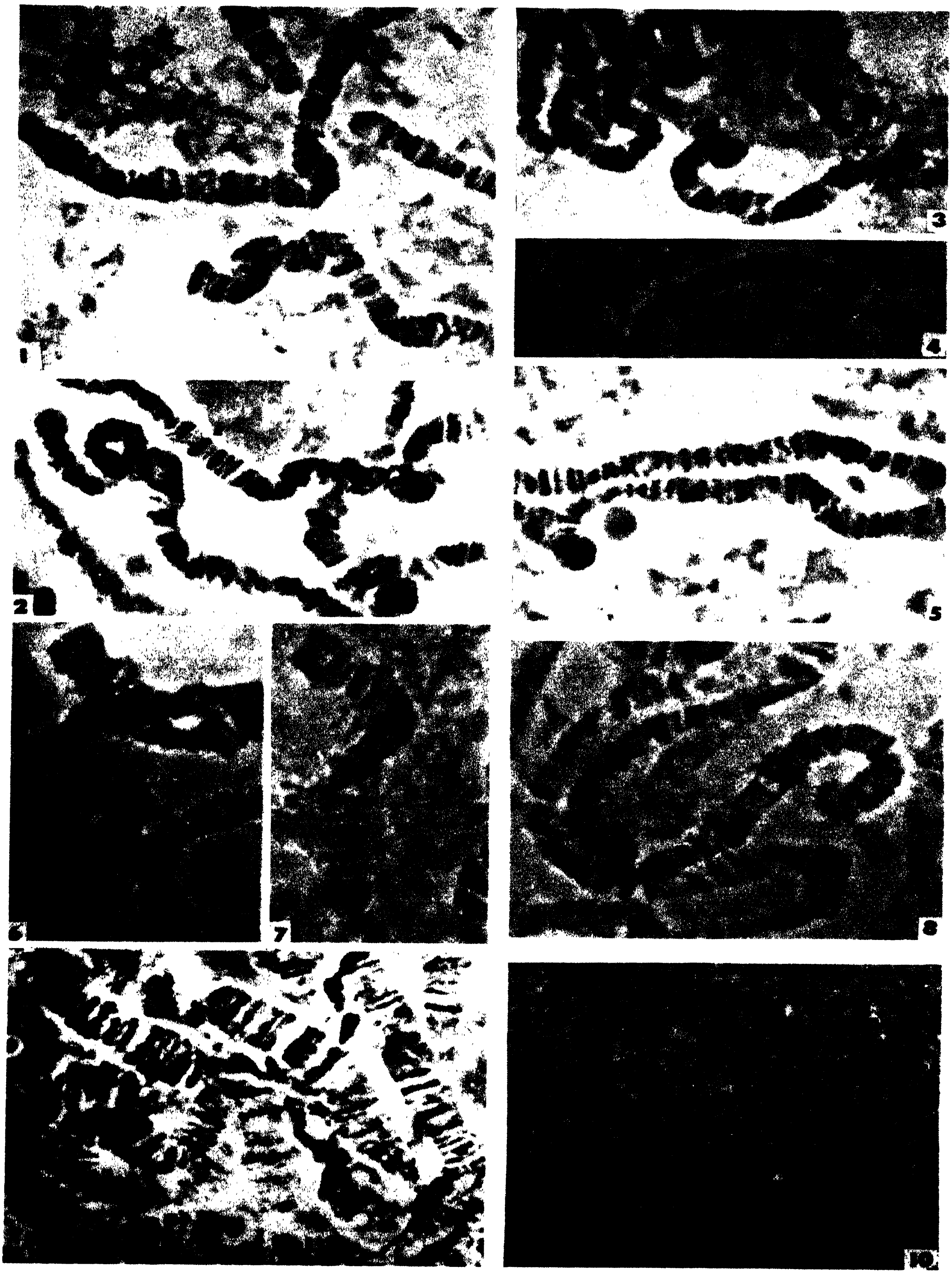

PLATE I (Schultz)—see reverse side for legends.

LEGEND TO PLATE I (see other side)

FIG. 1. Salivary gland chromosomes from a sc^8w^a male larva having a supernumerary Y chromosome (XYY). Note the cylindrical aspect of the chromosomes, and the compact structure at their ends. The arrow shows a nucleolus-like body, the "nipple" referred to in the text, on chromosome 2L. Aceto-carmine preparation. 2180X

FIG. 2. Chromosomes from a sc^8w^a male, on the same slide as that of Fig 1, but XY in composition. Note the ragged ends and the flatter aspect of the chromosomes. "Capsule" at arrow. 2180X

FIG. 3. XXY female: Note plumpness of chromosome and end at left with nipple (2L). At right is chromocentral region, with characteristic mesh-like structure. The supernumerary Y is seen at the arrow. Aceto-orcein preparation, counterstained with fast green; from same culture as individuals in Figs. 618. 2180X

FIG. 4. Chomosome end (2R) from XXY female, showing nipple-like body, taking the fast green stain as the nuclelous does. 2180X

FIG. 5. XY male, *gt* w^a, chromosomes from same preparation as Figs. 6 and 8 (XO males). Structure of chromosomes similar to those of Fig. 2, also XY male. Aceto-orcein with fast green counterstain. 3470X

FIG. 6. XO male, *gt* w^a: note ragged appearance of chromosomes, tendency towards dissaggregation of the bands, and flat ribbon-like structure. Ends are 3L and 3R. 3470X

FIG. 7. XO male, *gt* w^a: end of chromosome 2L, from another preparation. Chromosome flaccid, fibers in interchromomeres. 3470X

FIG. 8 XO male, from same preparation as Fig. 6. Note end of 3L spread out into a fan. 3470X

FIG. 9. Optical section of unfixed nucleus of *gt* w^a female salivary gland, photographed under the phase microscope. Note absence of capsules, even in chromocentral region (arrow). 2180X

FIG. 10. Phase contrast photograph of unfixed nucleus of XO male from same experiment as Fig. 6. Note end of chromosome 2L (arrow) flaring—compare Fig. 7; also ribbon-like structure of chromosomes, compare Fig. 9 Differences in contrast between Figs. 9 and 10 are not significant, since photographic conditions were not comparable.

These photographs were taken with the Spencer phase microscope equipment, using a 0.2 A-0.25 λ oil immersion lens, (N.A.1.25), 12x ocular. It is pleasant to thank Drs. E. K. Patterson and G. T. Rudkin for aid in the preparation of these figures.

in duplicate form and such duplications may well accumulate.

3. The cytochemistry of the giant chromosomes of Drosophila is discussed, in an attempt to find a diagnostic of heterochromatin. There are the four groups of substances present in chromosomal structures according to various tests (nucleic acids of the ribose and the desoxyribose type, filler protein-histone (?); and the elastic, fibrous protein of the interchromomere). Of these, the chief difference between heterochromatic and euchromatic regions is displayed by the elastic interband protein; this seems decreased in amount, or absent, in heterochromatin. The evidence as regards nucleic acids of the ribose type is still inconclusive, although to date no significant differences are evident between the different regions; the presence of distinctive ribose nucleic acid containing material associated with heterochromatin seems best interpreted as due to heterochromatin products, *e.g.*, nucleolar substance.

4. Consideration of the mutant genes found to be localized in heterochromatic regions also lends no support to the duplicate gene hypothesis, since although the bands with which these genes are associated behave as the other heterochromatic bands do in the giant chromosomes, the characters that they affect are very different.

5. The genetic properties of heterochromatin in Drosophila are discussed. Two characteristics appear: the production of a special type of male sterility, and the occurrence of variegation in euchromatin-heterochromatin rearrangements. The latter and very likely the former also are characteristic of the heterochromatic regions of each of the chromosomes, and are probably to be traced back to a few genes.

6. Both these properties of genes in heterochromatin are shown to be connected with effects on chromosome behavior. The sterility of the male results from failure of the sperm nucleus to differentiate properly. The effects on variegation are paralleled by a process of heterochromatization in the giant chromosomes, in which the euchromatic regions have a heterochromatic character imposed upon them as a position effect of the rearrangement.

7. An even more striking effect of the same kind is observed in the structure of giant chromosomes in individuals containing different numbers of Y chromosomes. As the number of Y chromosomes increases, the structure of all the chromosomes becomes better defined, as if the amount of chromosomal substance increased. This is believed to be in harmony with the view that the Y like other heterochromatin has to do with substances important in chromosome metabolism.

8. The nature of these substances is discussed in connection with the relation of heterochromatin to the nucleoli. Ribose-type nucleoproteins are concerned, providing a link with the cytoplasmic nucleoproteins. The theory that genes in heterochromatin have to do with precursor-substances of chromosome metabolism, and more generally speaking, of protein synthesis, is supported by these considerations.

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Discussion

Kaufmann: Dr. Schultz has indicated that heterochromatic regions are more sensitive to breakage by X-rays than are euchromatic regions. This statement applies if break frequency in Drosophila is determined in respect to salivary gland chromosome length. However, the extensive data that I have collected for *D. melanogaster* indicate that break frequency in both heterochromatic and euchromatic regions is proportional to the length that these regions occupy in the prophase chromosomes of somatic mitosis. It remains to be determined whether a similar condition obtains in the chromosomes of the mature spermatozoon at the time of irradiation. Nevertheless this possibility must be considered in any appraisal of chromosome structure in Drosophila based on the X-ray data.

Kurnick: Dr. Schultz stated that, judging from the staining of chromosomes with aceto-orcein and counterstaining with fast green after fixation, he has observed the removal of fast green staining material with buffers. I do not, by any means, wish to question his observation, but do wish to interject a note of caution into the interpretations based on fast green staining. I have recently developed a dye mixture consisting of aceto-orcein, fast green and salt which, after several combinations had been tried, provided a satisfactory stain according to the criteria of Dr. Ris, who served as judge and aided with the cytological material. This mixture, when applied to fixed or unfixed smears or squashed cells, stains chromatin a brownish red and nucleoli and cytoplasm pale green without differentiation. When we proceeded to use the original mixture of aceto-orcein and fast green without added salt in following our preparations of chromosomes, green staining material seemed to appear, disappear and reappear as we changed from one wash medium to another. Finally, it was observed that the chromatin threads stained quite greenish when suspended in a salt-free citric acid or acetic alcohol medium only to become reddish brown on the addition of salt. Washing the material free of salt again resulted in the reappearance of green staining material. We then observed that isolated nuclei suspended in acetic alcohol, when stained with fast green alone, stained quite intensely. On the other hand, if a drop of saline were added with the stain, the staining was very faint. It was thus apparent that fast green is very sensitive to the presence of salt, staining much less intensely in its presence than in its absence, and that the apparent appearance, disappearance and reappearance of fast green staining material, was due only to this varying intensity with which fast green stained in the presence or absence of salt.

The addition of salt to the aceto-orcein-fast green mixture has eliminated this difficulty so that the mixture now stains uniformly as described above: chromatin brownish red, nucleoli and cytoplasm pale green.

Schultz: We had observed in our own experiments, that the staining with fast green is contingent upon the buffer used. With phosphate buffer, for example, not even the bands of the salivary gland chromosomes stain well. In the experiments referred to, veronal buffers were used, under circumstances in which the loss of the material was clearly evident. Dr. Kurnick's interesting results provide the beginning of a possible analysis of the nature of the combination between the fast green and protein of the chromosome.

COMPOSITION OF CHROMONEMATA AND MATRIX AND THE ROLE OF NUCLEOPROTEINS IN MITOSIS AND MEIOSIS

J. A. SERRA

Data on the chemistry of the cell elements which have accumulated during recent years make it possible to draw a rough picture of such cytological events as mitosis and meiosis in more or less physiological terms, in contrast with earlier purely morphological descriptions and with the sometimes postulated "forces" of an unknown nature. Nucleic acids and nucleoproteins are among the constituents of the cell whose study has contributed most to an understanding of the phenomena involved in cell growth and division. It is therefore fitting to deal in the present Symposium with the role played by nucleoproteins and their components in mitosis and meiosis.

A discussion of the physiology of cell division would be fruitless without first considering the composition of the nucleus and especially of the chromosomes. Although this topic is treated in other contributions to the Symposium, it is necessary to clarify certain facts of cytological importance, particularly the distinction between the chromonemata and the matrix in relation to their chemical constitution. On the other hand, it could be feared that an overemphasis on nucleic acids and nucleoproteins might perhaps obscure the role played by other constituents in cell growth and division and that a division of biological problems into more and more specialized questions, though methodologically necessary, could result in viewing only the trees and not the forest. Accordingly, we propose to discuss also the functions of cell components other than nucleic acids and nucleoproteins, and to deal with certain general aspects of the discussed phenomena.

Techniques and Materials

The methods used in obtaining the histochemical data are the Feulgen reaction, the organic phosphorus reaction, color reactions with acid and basic dyes and digestions with nucleases (Serra and Queiroz Lopes, 1945a, b) for nucleic acids. For proteins, both general reactions such as the ninhydrin and arginine reactions and special tests, including the quantitative valuation of the arginine reaction and the tyrosine and tryptophane reactions, were employed (Serra, 1944, 1946). The lipids have been characterized by means of color reactions with the specific dyes Sudan III and BZL blue (Ciba) before and after digestion with protease (pepsin), with nucleases and with mineral acids and bases. The nucleases used were phosphatases extracted from rice bran with water and purified by precipitation with one volume of alcohol, again dissolved in acetate buffer of pH 4.0 and precipitated with an equal volume of alcohol. Nucleoproteins were extracted by buffers of suitable pH (carbonate-bicarbonate and phosphate, from pH 7.0 to 9.8) and by salt solutions of 1 M and about 0.2 M concentrations according to the method of Mirsky and Pollister (1943, 1946). A treatment with 0.2 N HCl after nuclease digestion was also used to extract basic proteins.

Cytological techniques include color reactions by the Feulgen method, by hematoxylin, methyl green, pyronin and light green and other usual dyes. Smearing of whole cells, and not microtome sectioning, was used throughout. The materials for the histochemical and cytological observations comprise several species of animals and plants, long and short horned grasshoppers, snails, salivary glands of Chironomus and Drosophila, frog ovaries and testes, onion and bean root tips, and bean anthers. The results obtained were always similar in all these materials, whose variety warrants the assumption that safe conclusions can be drawn.

Morphological Aspects of Mitosis and Meiosis

Before attempting to discuss the role played by certain cell components during nuclear division, it will be convenient to recall certain salient facts about the morphological aspects of the problem. In somatic mitosis there is a division of the chromosomes into two halves longitudinally and an orderly distribution of a set consisting of one half of each chromosome to the two cell poles. In meiosis there is also a division of each chromosome into two halves, but before this division is evident, the homologous chromosomes become paired; this is followed by distribution to each pole, not of a set of half chromosomes but of a set of chromosomes already divided in two halves. These two halves will separate in the second meiotic division, which regularly follows the first division. Meiosis is therefore a sequence of two mitoses with only one division of the chromosomes, these being apparently undivided (but see below) when pairing takes place in zygotene, while in ordinary mitosis the chromosomes are already double at the beginning of prophase.

During the prophase of mitosis and meiosis conspicuous changes in the chromosomes are observed. These changes are related to the peculiar condition of the resting stage nucleus before division. Mitotic chromosomes usually arise from resting nuclei of the thread type, that is from nuclei in which threads which take up chromatin dyes and give strong Feulgen and arginine reactions are conspicuous. Tissues composed of cells engaged in rapid mitotic divisions always show nuclei of this type. Meiosis, on the other hand, is connected with a phase in which the nucleus is of the "empty" or disperse type. Nuclei of this type seem almost optically empty, except for the nucleoli, or they may show points or blocks of chromatin of variable size, representing special chromomeres or heterochromatic regions. It is observed that meiosis usually begins after a period of intense multiplication by mitosis, at the end of which the nucleus is of the disperse type; an intense growth period is inserted during the meiotic prophase (particularly apparent in oocytes but also visible in spermatocytes and pollen mother cells). Meiosis is therefore related to the growth of cells which were previously engaged in active divisions.

Other important morphological facts of mitosis and meiosis concern the division of the chromosomes and their distribution to the two poles of the spindle. In morphological descriptions, the "division" of the chromosomes is the distinction at the microscope of two chromonemata where before only one was visible. It is obvious, however, that the visibility of two chromatids in a chromosome may be delayed beyond the true time of division, it being necessary that the two half-chromosomes be sufficiently far apart in order to be distinguishable microscopically. The determination of the time of division of the chromonemata may therefore give different results according to the materials and the methods of investigation. Experiments with X-rays, intended to give indication of the single or the double nature of the chromonemata, according to the production of chromosome or of chromatid breaks respectively, have yielded conflicting results (general summary in Sharp, 1943). The morphological and genetical evidence shows that the apparent "point of division" of the chromonemata is variable according to the object chosen and the method employed. This is to be expected if a variable condition determines the half-chromonemata separation; this condition will be shown below to be the matrix. The general conclusion is that chromosomes always behave as though they were double during mitosis and as though they were single at the leptotene and zygotene stages of meiosis. It is possible, however, that in some cases at least, the chromonemata are double at the very beginning of meiosis, in early leptotene (Kuwada, 1940, and references), but become single afterwards by fusion.

While the chromosomes are normally double bodies at the prophase of mitosis, the centromere or kinetochore behaves as though undivided until metaphase, when suddenly the two halves of each chromosome achieve their separation and the anaphase movement towards the poles begins. Morphologists are not yet certain whether the centromere is actually double or single during prophase, different methods giving conflicting pictures (see Darlington, 1939, and Schrader, 1944). The view which seems more probable is that chromonemata are double at the centromere region (primary constriction) but that the external envelope of the chromonemata at this point is single, as a kind of common sheath which divides only at metaphase.

As far as the spindle is concerned, it is obvious that it has little to do with the division of the chromosomes, as the existence of endomitosis and the failure of spindle function induced by colchicine, by other drugs and by physical agents demonstrate. The spindle is a mechanism of distribution of the chromosomes and to a certain extent of division of the cytoplasm. There is, however, a close relation between the spindle and the centromere, as is demonstrated by the movements of the chromosomes along the spindle fibers and by the fact that certain spindle fibers seem to extend between the centromere and the poles. Cases where instead of only one, well individualized, there are apparently "multiple" or "diffuse" centromeres (references in Schrader, 1944) show that the connections between chromosome and spindle fibers may be established at several points along the body of the chromosome, while in the majority of the actually known cases these connections tend to be especially localized in a given constriction of the chromosome. It is also possible that in these cases of "diffuse" centromeres the movements of the chromosomes may be brought about more by other forces than by the attraction by means of spindle fibers.

Composition of Chromonemata and Chromosomes

Nucleic acids

The Feulgen reaction demonstrates that the thymonucleic acid of the cells is localized only in the chromosomes. Ultraviolet photography shows beyond any doubt that the color reaction given by correct Feulgen test corresponds to nucleic acid, the intensity of the absorption at 2600 A being caused by the purine and pyrimidine rings present in the nucleotides. The question is different, however, with respect to other nucleic acids. As it is generally impossible or very difficult in cytochemical work to characterize the differences between all other nucleic acids, except thymonucleic, and since zymonucleic acid is the most important of the other acids, usually nucleic acids which do not give the Feulgen reaction are designated as ribonucleic. We will here adopt this simple nomenclature.

In chromosomes at a contracted stage it has not yet been possible to demonstrate conclusively the existence of ribonucleic acid. The color reaction given by the chromosomes with pyronin-methyl green in prophase and metaphase is different from the color reaction in other stages and this difference disappears after the action of ribonuclease (Brachet, 1941). Ribonuclease, however, is capable not only of attacking ribonucleic acid but also proteins, and under certain conditions (alkaline pH) thymonucleic acid is also lost from the chromosomes (Schultz, 1941). In salivary chromosomes we have obtained results in accordance with those of Schultz with pyronin-methyl green staining. The color is without doubt due not only to chemical affinity but also to adsorption, and is strongly influenced by the isoelectric point and the density of the structures. If ribonuclease attacks the proteins or thymonucleic acid, a changing of the isoelectric point and of the density of structure will result, even in the absence of ribonucleic acid.

We have made efforts to identify ribonucleic acid in the chromonemata of oocytes after a completely negative Feulgen reaction, by means of the phosphorus reaction coupled with digestion by nucleases. A faint blue color reaction was obtained, especially with fixatives that preserve the lipids, but this was probably due to the interfaces between nucleoplasm and chromonemata and is localized chiefly on points and small rods precipitated over the threads. We have not obtained any evidence of a differential distribution of ribonucleic acid or of another phosphorus compound on pure chromonemata from which chromatin has been removed, similar to that which is seen in salivary gland chromosomes for thymonucleic acid.

Thymonucleic acid seems also to be absent from the chromonemata of certain oocytes during the growth period. In snail and frog oocytes, after fixation by acetic-alcohol (1:3), which very well preserves the nucleic acid, a stage of oocyte growth is reached where the Feulgen reaction is completely negative on the chromonemata (Fig. 1). The diplotene chromosomes gradually lose their thymonucleic acid until after stage c of Fig. 1, and no more color with a Feulgen test is obtained, even when the chromosomes are gathered close to the principal nucleolus (in certain snails). Caution is necessary when employing formol containing fixatives, because this reagent is strongly retained by the tissue proteins and gives the aldehyde reaction.

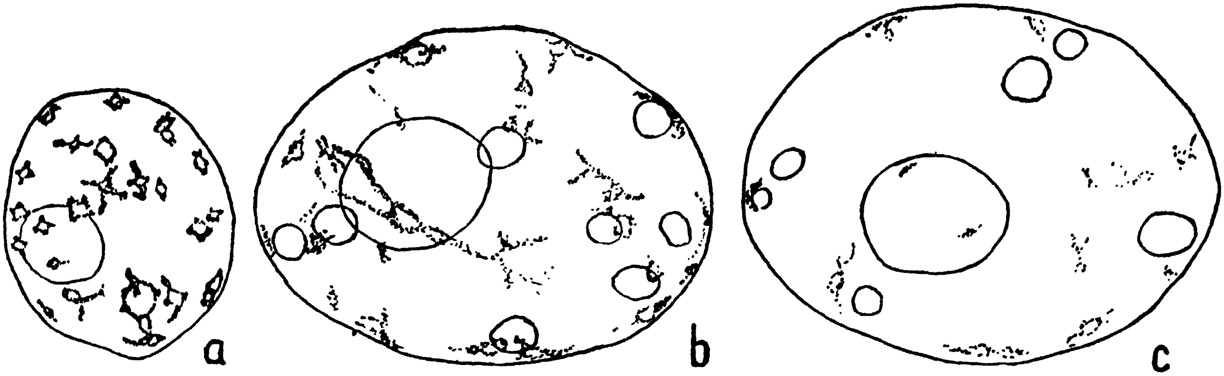

FIG. 1. Three stages of the distribution of thymonucleic acid in the nucleus of oocytes of the snail *Tachea nemoralis* L. Only the regions of the chromosomes attached to the nucleolus or to the nuclear membrane remain charged with thymonucleic acid until an advanced stage of growth. $\times$ 1750.

Basic proteins

If instead of the Feulgen reaction we use the arginine reaction, the results are similar in what concerns the existence of a charge of basic proteins, in contracted chromosomes. The reaction is very intense on the chromosomes in comparison with the cytoplasm. The contrast is particularly evident in animal spermatocytes in metaphase of the I meiotic division, when the chromosomes give a very intense reaction while the rest of the cell shows a very faint color. In plant pollen mother cells the cytoplasm (at least in the cases we have observed) is more rich in arginine than are animal spermatocytes. Except when there is a too high (protamines) or a too low amount of arginine, the reaction is approximately proportional to the concentration of this amino acid in the proteins, a very intense histochemical arginine reaction demonstrating the presence of a high amount of basic proteins (Serra, 1944b). It can be safely concluded that in relation to the cytoplasm the chromosomes in contracted phases possess a high amount of arginine-rich proteins.

In metaphase chromosomes of I spermatocytes (Figs. 13, 14, Plate II) the intensity of the color reaction is roughly equal to 4-6 times the intensity of the cytoplasm. Even allowing for the difference in density of the chromosomes in relation to the cytoplasm, which is perhaps 1.2-2.0 times greater, it is concluded that the chromosomes possess a great amount of arginine rich proteins. In somatic chromo-

somes the intensity of the reaction is also about 4 times that of the cytoplasm of old cells, but not of the cytoplasm of meristematic or young cells, which is also rich in basic proteins.

By treatment of the chromosomes with rice nucleases at pH 4.0 it is relatively easy to free the chromosomes from their nucleic acid. After fixation in acetic alcohol (1:3) the pieces are immersed in a nuclease solution at 38° C. Digestion of the nucleic acids is followed by means of the Feulgen reaction. When this reaction is negative the phosphorus reaction also becomes negative for the cytoplasm and the nucleolus, demonstrating that ribonucleic acid was also digested. By cytological color reactions the chromosomes are visible with apparently the same shape and size, but are difficult to stain because of the diminished density of structure. The arginine reaction is obtained with exactly the same intensity as before digestion, as the nucleases do not attack the basic proteins; in fact photographs 13 and 14 of Plate II could as well be taken after as before the digestion with nucleases.

The results of the reaction for arginine are particularly well shown on salivary gland chromosomes of Chironomus (in Drosophila the results agree, but Chironomus is more suitable material). The bands with thymonucleic acid give also an intense arginine reaction—Figs. 8, 9 and 10 of Plate I. These results will be dealt with in more detail below.

In chromonemata deprived of thymonucleic acid, that is, in oocyte chromosomes in middle and late second growth period, the arginine reaction is relatively less intense. A difference between the chromonemata and the materials which are deposited on them is particularly apparent in the leptotene and pachytene of meiosis. Hand in hand with the increasing charge of thymonucleic acid, the reaction for basic proteins is given with greater and greater intensity until the maximum is attained when the contracted stages are reached. Chromonemata without a charge of thymonucleic acid are also poor in basic proteins, the intensity of the reaction being only slightly superior to that of the old cells cytoplasm.

As the thickness of the chromonemata deprived of accessory materials lies within the limit of microscopic resolution, it is not possible to observe more details with the histochemical reactions. However, these data demonstrate without any doubt that it is not only thymonucleic acid but also basic proteins which deposit on the chromonemata, the metaphasic chromosome being formed by chromonemata with a heavy charge of thymonucleic acid and basic proteins.

Other proteins

Judging from the intensity of the arginine reaction given by oocyte chromosomes, the pure chromonemata are not formed, or are formed only in part, of basic proteins. As the chromonemata form a part of the metaphasic chromosome, it follows that there are proteins other than the basic ones in chromosomes in general. We have tried to separate these non-basic proteins from the basic by differential decomposition. After having treated fixed tissues (acetic-alcohol for one hour) of grasshopper testes with nucleases until a negative Feulgen reaction, the materials were extracted with 0.2 N HCl at 38° C for 2 days. After this extraction the tissues were refixed in acetic-alcohol-formol and subjected to the arginine and the tyrosine reactions. The arginine reaction was very feeble and it was not possible to observe any differential color reaction between chromosomes and cytoplasm. The tyrosine reaction was also feeble but the difference from the original condition of the materials was less noticeable.

The diluted HCl had dissolved the basic proteins especially but other proteins were also extracted, as shown by the marked shrinkage of the cells in general. Histones and other proteins have not very different amounts of tyrosine, so the reaction for this amino acid is characteristic of both basic and non-basic proteins and its intensity may be taken as roughly proportional to the total amount of protein present. Although these data are not conclusive by themselves, when considered with the histochemical results mentioned above for the chromonemata they point out that in chromosomes in a contracted stage there are, besides basic, also non-basic proteins in a smaller amount.

Chromosomes and pure chromonemata give a positive tryptophane reaction which is characteristic of basic and non-basic proteins. [Claims that histones do not possess significant amounts of tryptophane are perhaps due to faulty hydrolysis. Data by Mirsky and Pollister, 1943, indicating absence of tryptophane in histones, have recently been corrected (Mirsky and Pollister, 1946; see also Serra, 1947).] The results of the tryptophane reaction reinforce those obtained by means of the tyrosine test.

Lipids

It seems probable that a part of the nucleoplasm will be imprisoned within the chromosomes when they pass from the relatively loose state exhibited in early prophase to a more contracted one in metaphase. This inclusion of nucleoplasm in the chromosomes is particularly obvious in certain salivary gland nuclei, for instance those of Sciara, which are almost devoid of nucleoplasm and possess chromosomes with a kind of alveolar structure corresponding probably to nucleoplasm within the chromosomes (Metz, 1941).

The composition of the nucleoplasm is little known. It is certain that it possesses proteins and other components, but details are lacking. Recently it was shown by analysing isolated nuclei that there is a high amount of lipids in the nucleus, almost

equal to the general content of the tissues (Stoneburg, 1939; Williams *et al.*, 1945). The greater part of the lipids are phospholipids (90% in dog liver nuclei) and especially lecithin and cephalin (respectively 61.7 and 24.0% of the total lipids of the nuclei, according to Williams *et al.*, 1945). Although the nucleolus probably has lipids, it is not possible that all the lipids of the nucleus be within the nucleolus. The conclusion is reached, therefore, that the nucleoplasm has a content of lipids more or less similar to the contents of the cytoplasm, but with a different qualitative composition.

By histochemical tests specific for lipids, before and after partial digestion with mineral acids and bases and with proteases (pepsin) it was not possible to obtain a general coloration of the nucleoplasm with lipid stains. It is well known that the lipids combined with proteins are difficult to demonstrate by histochemical means. The negative results indicate that the lipids of the nucleoplasm are combined in lipo-proteins. Usually the nucleolus only gives a faint coloration with lipid dyes, suggesting a localization of the lipids chiefly at its surface. However, in certain nucleoli of growing oocytes there are peripheral inclusions which give a coloration of middle intensity with BZL blue. This observation, which we have made repeatedly on snail oocytes, indicates that the nucleolus also has lipids but these are usually impossible to demonstrate.

The chromosomes in contracted stages do not give a visible lipidic coloration. However, oocyte chromonemata are surrounded by a kind of lipidic "halo" after their charge of thymonucleic acid and basic proteins has disappeared. When examined under great magnification little dots and small rods surrounding the chromonemata exhibit a faint color reaction with specific lipid dyes, but as far as it is possible to make conclusive observations on structures so thin, the chromonemata themselves seem deprived of any color. The general conclusion reached is that chromonemata and chromosomes are not rich in lipids, but that a layer of these, forming a pellicle or figured particles may separate the chromosomes and chromonemata from the nucleoplasm. To the extent that nucleoplasm is within the chromosomes these should also contain lipids in their composition. It is of no use, however, to employ for the demonstration of chromosome and nuclear lipids unspecific tests such as colorations by Nile blue, osmium tetroxide, etc., which give hazardous results and must be confirmed by specific tests.

Interchromomeres, Chromomeres and Genes

In chromosomes at the beginning of meiosis it is possible to recognize the existence of a kind of small beads alternating with thin fibrils. The beads give a positive Feulgen reaction, while the threads between them may show only a faint color reaction or be colorless. Chromosomes of salivary glands of Dipteran larvae give a similar picture; along the chromosomes bands with a heavy charge of thymonucleic acid alternate with interbands completely negative with respect to a Feulgen reaction.

The results are similar if the arginine reaction is employed. The bands of the salivary chromosomes give an arginine reaction with an intensity approximately 4 times that of the interbands, which corresponds to the existence of a great amount of basic proteins in the bands, while the interbands are deprived of these proteins or possess them in small amounts (Serra and Queiroz Lopes, 1944). (Figs. 8, 9 and 10 of Plate II.) The digestion of the nucleic acids with acid phosphatases (but not with alkaline ones, which decompose the basic structure of the chromosome) leaves the chromosomes apparently untouched as far as their basic proteins are concerned; the arginine reaction is given with the same intensity before and after the Feulgen reaction has become completely negative. On the other hand, it is very difficult to observe the distribution of the basic proteins in leptotene chromosomes because the chromonemata are very thin, but in favorable cases a difference in intensity is seen between beads and fibrils, corresponding to the picture obtained by the Feulgen reaction.

The beads of the leptotene-pachytene chromosomes and the bands of the salivary gland chromosomes may be called *chromomeres,* while the fibrils and the interbands respectively are the *interchromomeres.* This common designation is necessary for descriptive purposes but does not imply that the two structures are strictly homologous. In effect, the salivary gland chromosomes are several times longer than the leptotene-pachytene chromosomes and therefore beads which in these latter seem simple may resolve into several bands in the salivary chromosomes; that is, in relation to salivary chromomeres, meiotic chromomeres may be either simple or more usually multiple chromomeres.

A considerable body of data gathered chiefly on salivary but also on meiotic chromosomes may be interpreted by the hypothesis that the genes are located on the chromomeres. However, the effects of rearrangements may complicate the precise location of the genes on a limited region of a chromosome and in some cases the limits of the gene seem imprecise and variable (Demerec, 1941). Goldschmidt (1945 and former publications) constructed a theory based on these effects of rearrangements, according to which there are no particulate units of heredity, or genes, and mutation consists in a change of the chromosome structure.

On the other hand, the existence of chromomeres in meiotic chromosomes and the differences between bands and interbands in salivary chromosomes has been interpreted as being due to spiralization of the chromonemata, the superposition of the gyres

giving rise by optical illusion to the appearance of beads and bands respectively (references in Ris, 1945). So-called lampbrush chromosomes of animal oocytes have also the appearance of continuous chromonemata with beads, but by careful observation we have always been able to resolve the bead-like appearances into gyres of a helicoidal coiling of 4 chromonemata, the "hairs" corresponding to rod-

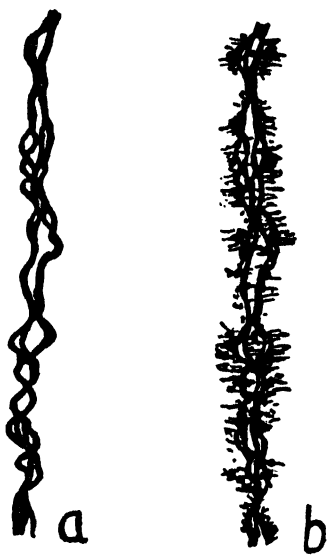

FIG. 2. Structure of lampbrush chromosomes. The "hairs" are apparently caused by chromonemata coils (*a*) and by precipitation of rod shaped particles of the nucleoplasm on the chromonemata (*b*). Stained with iron hematoxylin.

like and bead-like structures of the nucleoplasm deposited on the chromonemata and also, in a lesser degree, to gyres of the chromonemata themselves (Fig. 2).

As was said above, by several tests for nucleic acids, proteins and lipids it was not possible to reveal in "pure" oocyte chromonemata an alternation of bands and interbands similar to what is found in salivary chromosomes. This negative evidence is not conclusive, however, as it is possible that if a difference did exist it could be inferior to the sensitivity of the tests employed. This important problem may actually be approached only in an indirect manner by means of the salivary chromosomes. We have treated salivary gland chromosomes with extractives of nucleoproteins and of proteins in general in order to characterize the residue of the bands. Chironomus salivary glands fixed in acetic alcohol (1:3, for 1-2 hours) were extracted at 38° C. with 1 M NaCl until the Feulgen reaction was negative or almost negative. After this the glands were refixed and the arginine and tyrosine reactions executed. The arginine reaction revealed that the differential color reaction given by the bands had disappeared completely or almost completely. Nevertheless, the bands still persisted and were visible after staining with acidic stains, but their color was similar to that of the interbands. The tyrosine reaction, which before the treatments was a little more intense in the bands than in interbands (Fig. 11 of Plate I), gives after the extraction of nucleoproteins a uniform color. Results with treatments by buffer solutions of pH 8-9 are similar if the extraction is not too much prolonged. Alkaline solutions tend to dissolve not only thymonucleic acid and basic proteins, but also other proteins of the chromosomes, resulting in salivary chromosomes in a decomposition into fibres and dot-like structures and in mitotic chromosomes in a kind of empty body with a Feulgen positive pellicle and core (Fig. 15, Plate II). All these results are explained by the assumption that in the bands there are also non-basic proteins.

The data so far existent is in favor of the conclusion that the bands of the salivary chromosomes correspond to the beads of the meiotic chromosomes in having a charge of thymonucleic acid and basic proteins on certain points of a continuous structure of non-basic proteins, which exist all along the chromosome (Fig. 3). If this interpretation is ac-

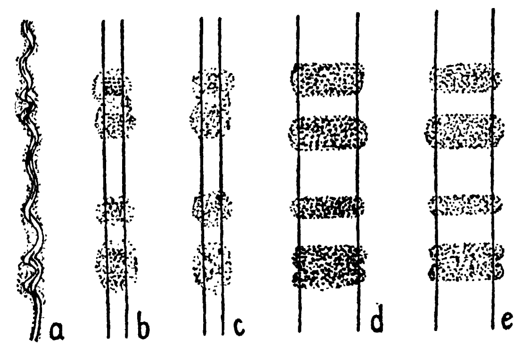

FIG. 3. Chromonemata of oocytes (*a-c*) and salivary chromosomes (*d, e*). In *a* a double chromonema with a small charge of peripheric nucleoproteins (dotted) and a differential spiralization at certain points where the nucleoprotein charge is heavier. *b* and *c* two alternatives hypotheses of the composition of the chromonemata; in *b* nucleoprotein regions (heavy dots) alternate on the chromonema with simply protein regions and in *c* it is the deposition of peripheric nucleoproteins (light dots) which causes the apparent alternation of chromomeres and interchromomeres. *d* and *e* two similar interpretations for salivary chromosomes.

cepted, then the appearance of beads in meiotic chromosomes and the formation of bands in salivary chromosomes are to be considered a consequence of a differential deposition of thymonucleic acid and basic proteins on certain points of the chromonemata, the spiralization becoming also more tight on these points than in the interchromomeres. This question is also important with respect to the nature of the gene and will be discussed in more detail

under the heading "The role of nucleic acids and nucleoproteins in mitosis."

Distinction between Chromonemata and Peripheric Nucleoproteins

The facts we have referred to above, though only of a qualitative chemical order, permit some conclusions to be drawn about an important distinction between chromonemata and chromosomes. It is only in oocytes and to a lesser degree in certain types of resting stage nuclei, namely in premeiotic nuclei of the disperse type, that the chromosomes are reduced to their essential elements. In nuclei of actively dividing tissues, in all the phases of mitosis and meiosis and in many nuclei of cells engaged in secretion and other physiological activities, there are chromatic threads which give the Feulgen and arginine reactions due to their possession of thymonucleic acid and basic proteins. It is to be expected that at least a part of the nucleic acid and basic proteins be combined in salt-like linkages, forming nucleoproteins.

Besides the evidence brought to bear upon this point by chemical research on components isolated from the tissues, which will be discussed below, the conclusion that in chromosomes the nucleic acid and the basic proteins are combined derives from the fact that the isoelectric point of the cell structures is approximately neutral. It should be otherwise if the nucleotides with their phosphoric acid groups, and basic proteins with free amino groups were not combined with one another. It is therefore highly improbable that a great part of these compounds be uncombined.

We have named the compounds which deposit on the chromonemata the *peripheric nucleoproteins,* which correspond more or less to the matrix or kalymma of the morphologists. This term of matrix has now fallen almost into disuse and most authors seem to postulate that the chromosomes are the chromonemata plus a charge of nucleic acid. This is easily seen to be untrue, as the results of the arginine reaction demonstrate. On a strictly morphological basis it is therefore legitimate to call matrix, or better kalymma, the materials which deposit on the chromonemata, but it is more precise to designate them as peripheric or kalymmatic nucleoproteins. It must also be remembered, however, that in many cases the matrix of the morphologists was an artificial appearance produced by defective fixation or other treatments. To sum up this point we may conclude that the chromosomes are composed of chromonemata, the permanent structures which must carry the genetic materials, and accessory components, mainly nucleoproteins composed of thymonucleic acid and histone-like proteins; these nucleoproteins, though important in cell physiology, are not necessarily related to the genetic activity of the chromosomes.

The Role of Nucleic Acids and Nucleoproteins in Mitosis

It is always difficult to pass from a static analysis to a dynamic synthesis. This is particularly true of the role played by the nucleoproteins and their components during cell division, since our data on the composition of the nucleus are of a fragmentary nature and do not cover the various stages of mitosis. It would be possible to analyse nuclei in successive stages of division by isolating cells in comparable phases, for instance the cell groups of certain grasshopper testes.[1] As long as such results are not available, the conclusions on the dynamics of the chromosomes will rest upon basic conceptions not necessarily extending from the experimental data. We will try in the following to discuss the functions of nucleoproteins and their components with a minimum of recourse to *a priori* hypotheses.

Are the chromonemata composed of nucleoproteins?

The first question to be discussed is the composition of the chromonemata in their "pure state." As revealed by the histochemical tests, certain oocyte chromonemata are composed of proteins which are chiefly (or exclusively?) of non-basic type. Nuclei acids are not demonstrable by the methods we have at present, at least in chromonemata of grown oocytes of snails, insects and frogs. Other workers have also envisaged this question and the results are variable according to the materials and the techniques employed (references in Brachet, 1944, p. 67-71). Only the cases where the so-called chromomeres are very thin and demonstrable to be optical effects of the helicoidal coiling, are of interest for the discussion of this point, since the presence of nucleoproteins in the bead-like structures causes a differential coiling and a differential reaction for thymonucleic acid, similar to that of leptotene chromosomes. If acetic alcohol (1:3) is employed and the necessary precautions are taken, it is certain that the Feulgen reaction becomes completely negative when the oocytes of several snails, of frogs and several Orthoptera are sufficiently grown. With formol-containing fixatives the results are dubious, because this aldehyde combines with certain groups of the proteins (probably with amino and imino groups). It is necessary to avoid aldehydes in the fixative, and caution is necessary even with common alcohol, which also has aldehydes.

[1] In Tettigonia, for instance, the testes show cell groups in the same stage of mitosis and of meiosis. It would be a matter of skill and patience to isolate these aggregates after fixation by freezing-drying or other suitable technique and to analyse them with respect to the principal components. It would also be feasible to isolate chromosomes from this material. We hope that such a work will be performed in American laboratories, our own resources in personnel and means being too limited to carry on such a program of research within a reasonable time.

Despite these precautions and the negative results, it is always possible to suppose that thymonucleic acid is in fact present in an amount below the sensitivity of the reaction employed. Theoretically it has been admitted that the genes are nucleoproteins and therefore the chromonemata, where the genes are located, should also possess always nucleic acid. This acid could be ribonucleic or thymonucleic, the first being also present (or nucleotides similar to ribonucleic acid) in viruses. It would be interesting to build hypotheses on the fact that ribonucleotides, which seem less apt to polymerize and to form fibres, belong to the elementary self-reproducing particles of viruses and of the cytoplasm, while thymonucleoproteins, with their tendency to form fibres, give rise to chromosomes. However, the cytochemical data we have up to the present do not furnish sufficient basis for a final conclusion. This important point may be approached by chemical analysis of isolated pure chromonemata; work is now in progress in our laboratory in this direction, but the results as yet are not conclusive. It should also be mentioned in this connection that according to some workers the virus-particles usually isolated could be only a part of the virus body, perhaps its reproductive units, homologous to the gametes of cellular organisms (Bawden, 1945). Indeed, the role of nucleic acid in viruses could be in the same relation to their reproduction, as the nucleoproteins of the chromosomes are in relation to the division of these bodies.

Another point with a bearing on this question is the composition of salivary chromosomes. As said above, there is no doubt that bands rich in thymonucleic acid and basic proteins alternate with interbands deprived of thymonucleic acid and of other nucleotides (Caspersson, 1940, give the accuracy of the method as 5%) and also with less (or no?) basic proteins. The results of extractions with NaCl and alkaline solutions are in favor of the existence of a continuous skeleton of non-basic proteins upon which nucleoproteins composed of thymonucleic acid and histone-like proteins are firmly bound at certain points, the chromomeres or bands. (It seems to us that it is not legitimate to suppose that proteins similar to protamines run along all the salivary chromosome, as suggested by Schultz, 1941. This was based apparently on a statement of Caspersson, 1940, referring to an absorption in the ultraviolet below 2600 A due to aliphatic amino acids. These amino acids are not characteristic of protamines.)

The other alternative is that along the salivaries bands and interbands interrupt the chromosome, like a pile of two kinds of coins, say silver and gold, alternating. Although we have formerly been a partisan of this hypothesis it seems to us now that it appears less likely when the facts about a continuous structure running all along the chromosome are taken into account (see also Mazia, 1941). When compressed salivary chromosomes resolve into fibrils, the arginine reaction given by these fibrils is more intense than that of the kind of cement which is seen between the fibrils, but there are no indications that the fibrils consist of basic proteins, though they may have a greater concentration of arginine, probably due to their being more dense than the cement between the fibrils (Fig. 9, Plate I). The cement probably is related to the nucleoplasm. On the whole, the facts we know at present, though not excluding completely the other alternative, are rather in favor of the continuity of proteins not rich in arginine and the location of nucleoproteins composed of thymonucleic acid and histones on certain points believed to be connected with specific genetic activity.

Functions of the peripheric nucleoproteins

Individualization of the chromosomes. Nuclei engaged in mitoses never show their chromonemata deprived of peripheric nucleoproteins and in embryonic and physiologically active tissues the so called resting stage is characterized by the nuclei also having chromatic threads corresponding to the chromosomes. The Feulgen reaction indicates that the amount of thymonucleic acid increases during prophase; according to Caspersson (1941) this increase takes place chiefly at the beginning of prophase. We have also remarked an increase of the coloration given by the arginine reaction at the beginning of prophase and especially close to the nuclear membrane (Serra and Queiroz Lopes, 1944). It may be concluded, therefore, that thymonucleotides and basic polypeptides come from the cytoplasm through the nucleoplasm and deposit on the chromatic threads, increasing their visibility and staining properties. This individualization of the chromatic threads is one of the functions of the nucleoproteins during mitosis and in the interphases.

Contraction of the chromosomes. Another role of the nucleoproteins is associated with the contraction of the chromatic threads which permits the regular distribution of the chromosomes to the middle of the spindle and afterwards to the two poles. The chromatic threads of mitosis and meiosis always exhibit some degree of coiling into one or more helices (in meiosis a "minor" and a "major" spirals may be visible). Great importance has been attributed by most morphologists to this coiling, which is believed to represent one of the principal characteristics of the chromosomes. However, it is easily seen that the coiling is sometimes only an expression of the impossibility of distention of the threads in a limited space; the three dimensional helicoidal coiling is rather a natural form of adaptation to the conditions prevailing within the nucleus.

At certain stages of the division cycle the helicoidal coiling becomes very tight, with a helix of small diameter, and in ordinary mitotic chromo-

somes it is very difficult to observe it in intact nuclei. By treatments with weak alkaline solutions the chromatic threads elongate and the helix uncoils, metaphase chromosomes giving rise to a kind of prophase nucleus (for instance with a treatment by ammonia and chloroform vapors, Wada, 1939). It is also well known that the treatment with weak alkaline solutions before fixation favors the observation of spiralization in histological preparations. It must be concluded, therefore, that the tightness of the helicoidal coiling in metaphase when compared with prophase is related to the existence of certain compounds—the nucleoproteins—which are more or less removed by the treatments.

A result similar to that obtained with alkaline solutions before fixation may be obtained by extracting nuclei with 1 M NaCl after fixation for a short time in acetic-alcohol. This method gives particularly interesting results in plant material, while animal tissues are more sensitive to fixation. When the nucleoproteins are almost all dissolved (extraction followed by means of the Feulgen and arginine reactions) the chromosomes show their chromonemata with a small amount of nucleoproteins and it is easy to observe the helicoidal coiling even in metaphase of ordinary mitosis. (Owing to the very faint coloration it is difficult to obtain good photographs of these chromosomes but the figures seen at the microscope are conclusive.) After an extraction of the nucleoproteins, prophase and telophase nuclei show a striking contrast; while the former have finely waved, more or less uniform chromonemata (divided in two), the latter typically exhibit the aspect of beads and fibrils which is usually seen in the meiotic prophase (Figs. 17 and 18, Plate II). In smears of advanced telophases of *Allium cepa* root tips, it is possible to see that the chromonemata are divided and the two halves spiralized, with bead-like structures (Fig. 18). These chromonemata look very similar to the pachytene pairs of meiosis; the beads are not resolvable into spiral gyres, at least the larger ones. Besides providing a means for the distinction between prophase and telophase, these results of extraction with NaCl prove that the state of the peripheric nucleoproteins is different in prophase and telophase. While in prophase these compounds are all bound to the chromonemata with about the same strength except the outermost layers; in telophase the external part of the nucleoproteins is easily soluble but the chromonemata maintain a thin layer of these compounds firmly bound. The existence of beads must have the same meaning as in the prophasic chromomeres of meiosis and is certainly related to a differential spiralization.

From all these observations it follows that excepting the residual coiling seen even in oocyte chromonemata, the so-called spiralization in all of the stages of mitosis and meiosis very probably is causally related to the deposition of peripheric nucleoproteins on the chromonemata. This is easily understood if the properties of thymonucleoproteins are considered. In effect, it is well known that these proteins are highly viscous gels with fibrous properties; for instance they adhere strongly to glass rods when their solutions are being stirred (Mirsky and Pollister, 1943). To explain the contraction of the chromosomes it is sufficient to admit that the nucleoproteins depositing on the chromonemata are progressively gelating by aggregation of the particles and dehydration. Spiralization must be caused by the colloidal properties of the peripheric nucleoproteins. When sufficient gelation has taken place, for instance in the more lengthy prophase of meiosis, or under the action of dehydration agents (cold and heat treatments) the chromosomes will contract more strongly, tending to assume a spheroidal form, which is practically attained in many cases at meiotic diakinesis.

Another point in this connection is the role played by each of the two main components, thymonucleic acid and histone, in the colloidal properties of the peripheric nucleoproteins and of the chromosomes. Certain cytological observations such as the so-called "diffuse" stage of diplotene chromosomes in some animals and perhaps also the "starved" chromosomes obtaining after cold treatment (Darlington and LaCour, 1940) suggest that histones by themselves, or with only a small quantity of nucleic acid, are related with a less strong spiralization. We have observed that snail diplotene chromosomes in a diffuse stage show also a less intense arginine reaction, but the charge of basic proteins seems not to have diminished as strongly as the thymonucleic acid. Nucleic acid and proteins tend to be disposed at the periphery of the chromonemata in hair-like protrusions, as a kind of atypical lampbrush chromosomes; in fact, the diffuse stage is the homolog of the lampbrush stage of the oocytes.

Owing to the lack of response of several animal and plant species we have subjected to the cold treatment, we have not yet observed what happens with starved chromosomes with respect to the intensity of the arginine reaction. From the morphological aspect it seems probable that basic proteins are less affected by the cold treatment than thymonucleic acid. The two components must have a certain independence of action but the full effect seems to depend on the presence of both in combination. Basic proteins alone are probably responsible for a lower degree of spiralization, while thymonucleic acid induces a stronger coiling. Future work on the colloidal properties of complexes of thymonucleic acid and histones, and other proteins, will permit a further analysis of the morphological changes involved in the prophasic shortening of the chromosomes. It seems almost certain that at the end of prophase not only the matrix but also the whole chromosome with its chromonemata becomes more gelate in relation to its state at the beginning of mitosis.

Division of the chromosomes. Besides contributing to the individualization and the shortening of the chromosomes, nucleoproteins certainly have an additional role to play in nuclear division, namely their role in the division of the chromosomes. The morphological division of the chromosomes is no more than an individualization of two chromonemata by their peripheric nucleoproteins. This role in division is an extension of the role played in the individualization of the chromatic threads.

Prior to the separation of one chromonema in two halves, a multiplication of the basic structures of the chromonema must necessarily have taken place, that is, a reduplication of its proteins (or nucleoproteins, if the hypothesis of the nucleoprotein nature of the gene be accepted). This reduplication very probably occurs during the interkineses, since the growth of the cell usually ceases or slows down during the middle stages of mitosis. The so-called division of the chromonemata in mitosis should consist of the individualization of two bundles of

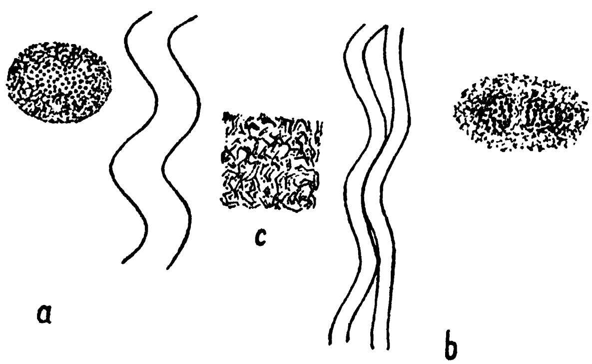

FIG. 4. Role of the peripheric nucleoproteins in the "division" of the chromonemata. A chromonema, with its protein structures already reduplicated (*a*), is split into two halves separated by peripheric nucleoproteins. The division is apparent after peptization of the superficial layers of peripheric nucleoproteins between the two chromonemata (*b*). In *c* a scheme of the constitution of the peripheric nucleoproteins, with nucleic acid and basic protein long particles oriented in all directions.

already reduplicated structures. The phase during which it is most probable that the two halves become sufficiently individualized, that is, charged with peripheric nucleoproteins distinctly separated from those of the other half-chromonema, is the anaphase-telophase. In fact, the charge of nucleoproteins has just attained its maximum and the gelation process is now reversed, a process of peptization then beginning which will permit the distinguishing of the two halves (Fig. 4). The innermost layers of the peripheric nucleoproteins are more or less decomposed while the part of these compounds attached to the chromonemata is more firmly retained, as was said above, and this causes a distinction between the two halves. It is in this stage that fixatives and the treatments to which the tissues are subjected may induce a more or less easy observation of the "division" (Delbrück, 1941). The variability of the results found in different materials and by different observers is attributable not only to differences in the treatments but also to the natural variation of the peptization process of the peripheric nucleoproteins, upon which the microscopic visibility of the individualism of two sister-chromatids depends.

If these conclusions about reduplication of the chromonemata and individualization of two halves be accepted, it follows that it is not legitimate to conclude that a lack of division of the chromosomes has occurred, by merely observing the microscopic aspect. Two variables must always be considered in this connection: the reduplication of the chromonematic protein bundles, and the role played by the peripheric nucleoproteins. These considerations apply also to meiosis, where the division of the chromosomes is generally seen only after the chromonemata are heavily charged with nucleoproteins and a mild peptization at the passage from pachytene to diakinesis is taking place. In some instances it appears that a separation of half-chromonemata which took place at the previous telophase is visible in the leptotene of meiosis. It is even possible that this is the general case, but that the relative lack of nucleoproteins prevailing at the onset of meiosis does not permit that the individualization of the half-chromonemata be maintained and a partial fusion of the two halves will therefore result. The same applies to salivary chromosomes. Here the relative lack of peripheric nucleoproteins does not hinder the reduplication of the protein structures of the chromonemata, since these grow enormously, but distinct chromosomes are not formed and the four chromatids of the two homologous chromosomes become more or less intimately fused owing to homologous attraction.

THE ROLE OF THE NUCLEOPROTEINS IN MEIOSIS

As in ordinary mitosis, the nucleoproteins have in meiosis the role of individualizing and "dividing" the chromosomes and also of contributing to the helicoidal coiling of the chromonemata. Moreover, the peculiar distribution of the nucleoproteins on the chromonemata at the beginning of meiosis certainly is connected with the essential characteristic of meiosis—homologous pairing.

Homologous pairing

Among cytologists it is generally accepted that the homologous pairing of meiosis is the consequence of the chromonemata being then single, while in ordinary mitosis the double nature of the chromonemata allows the forces of attraction between the two halves of each chromosome to be satisfied. Meiosis has been related to the lack of divi-

sion of the chromosomes, in the precocity theory of meiosis of Darlington, in which it is supposed that meiosis is a mitosis prematurely begun while the chromonemata are yet unreduplicated. The forces of attraction give place to forces of repulsion after the chromosomes divide at the end of pachytene.

The relative lack of nucleoproteins. Indications about the nature of the process of homologous pairing are given by the attractions manifest at other stages, namely in the mitoses of Diptera, in salivary chromosomes and in heterochromatic chromosomes. The first is known as *somatic pairing* and is exhibited from the end of prophase until metaphase (Hinton, 1946) in all tissues of the Diptera so far examined in this respect. The same phenomenon is expressed more fully in salivary gland nuclei by a fusion of the homologous chromosomes. Homologous pairing in both ordinary chromosomes and salivaries of the Diptera appear connected with a relative lack of nucleoproteins. It is familiar to every observer of Dipteran chromosomes that they seem to suffer from a chronic lack of distinctness when colored by chromatin dyes or a Feulgen reaction. This lack is particularly apparent during the ordinary mitotic prophase, resembling more or less closely what happens at the beginning of meiosis in other groups. It seems probable, therefore, that somatic pairing derives from a peculiar metabolism of nucleoproteins in the Diptera which makes these compounds relatively unavailable during all the mitoses and also during the endomitotic reproduction of the chromosomes in salivary gland nuclei.

On the morphological side, this lack of nucleoproteins corresponds to a distinctness between chromomeres and interchromomeres. In Dipteran somatic chromosomes it is not easy to observe this distinctness, but it is seen in salivary chromosomes and in meiotic chromosomes of many species. Cases more or less intermediary between mitosis and meiosis also occur in apomictic plants (Gentcheff and Gustafsson, 1940). It seems that it can be safely generalized that a homologous pairing, be it somatic or meiotic or in salivary chromosomes, is the consequence of a relative lack of nucleoproteins.

This lack probably derives from the somatic multiplication of the gonial cells before meiosis at a too rapid rate. In testes it can be observed that meiosis does not set in abruptly, but on the contrary, that the spermatogonial divisions gradually become more "clear" from a staining point of view, and slower, as if the charge of peripheric nucleoproteins was decreasing (particularly clear in Orthopteran testes). At the same time, the cytoplasm increases and the whole cell as well as its nucleus is markedly larger when meiosis begins. Meiosis is thus related to growth and seems to be the result of a balance between growth and multiplication; the place occupied by the cell may exert an influence through the quantity of nutrients available.

This relation between growth and multiplication can be established by means of the nucleoproteins, since it is known that actively growing cells are rich in nucleotides and in basic proteins. The phenomena are particularly evident during the growth of the oocyte. While the oocyte cytoplasm is increasing, the nucleus loses almost all its nucleoproteins, at least as far as the chromosomes are concerned, and the nucleolus reproduces actively by budding, many of the nucleoli so produced being afterwards disintegrated in the nucleoplasm (Serra and Queiroz Lopes, 1945). A movement of the chromosomes and the nucleoli towards the nuclear membrane is clearly seen at the beginning of the more intense period of growth. This movement is also characteristic of the end of the meiotic prophase in cells where growth is less evident than in oocytes, giving rise to the well known scattering of the dyads in diakinesis; it must always result from metabolic currents directed from the nucleus towards the growing cytoplasm. It is also possible that forces of repulsion play a role in dispersing the bivalents through the nucleus but it is more reasonable to admit that the movements of the chromosomes, and that of the nucleoli observed in oocytes, is a response to the same factors.

In the case of oocytes it is obvious that there are not enough nucleoproteins for an intense growth and at the same time an intense supply of nucleoproteins to the chromosomes. Only after the elaborations of the cytoplasm and reserves come to an end can the metabolic currents carry nucleic acid and basic proteins (or more probably, their respective components, nucleotides and certain polypeptids particularly rich in arginine) to the chromosomes. Probably thymo and ribonucleotides are interconvertible in the oocyte and early embryonal life (discussion in Brachet, 1944) and the nucleoplasm of the oocyte must necessarily contain ribonucleotides, since the nucleoli decompose there during the growth period.

The conclusion, that meiosis results from a special balance between the nucleoproteins available to the cell and the growth of the cell conditioned by the elaboration of other proteins, implies that it will be possible to influence the onset of meiosis by means of upsetting the balance between nucleoproteins and proteins towards lower values than are found in actively dividing cells. Scarcity of phosphorus and other materials essential for the synthesis of nucleotides and arginine rich proteins would be a means of controlling the balance. It is to be hoped that by the use of isotopes sufficient knowledge about the synthesis of nucleotides and the possible relations between their synthesis and that of the basic amino acids will soon be available; without this knowledge it is not possible to project crucial experiments on this question. Speculatively it may be supposed that

the factors controlling the onset of meiosis will act upon the steps common both to the synthesis of nucleotides and of basic amino acids.

Salivary cells have a cytoplasm rich in basic proteins and nucleotides. An arginine reaction executed on entire cells and not on isolated chromosomes gives a very intense color reaction in the cytoplasm which makes difficult the observation of the chromosomes. A color reaction with pyronin, which essentially corresponds to the content of ribonucleic acid (Brachet, 1944 and former publications) shows that ribonucleotides are present in large amounts in the cytoplasm of the cells. This color reaction disappears after an extraction with physiological saline, which dissolves ribonucleoproteins (Mirsky and Pollister, 1946). These data demonstrate that in salivary cells the balance of the nucleoproteins is upset in favor of the cytoplasm, the nuclei remaining relatively free of nucleoproteins. Probably the high amount of nucleoproteins in the cytoplasm is related to the secretion effected by the salivary cells. The relative lack of nucleoproteins in salivary cell nuclei persists during the entire development of the larva and this permits a fusion of the chromosomes, while in ordinary Dipteran cells somatic pairing does not give rise to an intimate pairing because at the end of prophase the charge of nucleoproteins of the chromosomes rapidly increases and the chromosomes separate.

The mechanism of pairing. The conclusion, that the balance of the nucleoproteins is very probably a decisive factor determining the onset of meiosis, has some immediate consequences which are important for an explanation of the meiotic pairing. In effect, if the nucleoproteins are necessary for the individualization of two half-chromonemata from a single one, it follows that at the beginning of meiosis such an individualization is not possible, or if it were begun in the previous telophase it would retrogress, causing the leptotene chromosomes to appear in general single.

Another consequence of the special balance of nucleoproteins is the differentiation of chromomeres and interchromomeres which is characteristic of the meiotic prophase and of salivary chromosomes. This differentiation appears to be necessary for an intense homologous pairing, while a partial fulfilment of it probably results in somatic pairing. Chromosome regions with an excess of nucleoproteins, that is, ***heterochromatic regions,*** also show a kind of pairing at meiosis but it is observed that this pairing is not strictly homologous and resembles a general attraction. Even in these regions, however, a scarcity of nucleoproteins and a partial differentiation in chromomeres is observed in leptotene-pachytene.

In what concerns the mechanism of pairing it is necessary to admit that long range forces are involved, since it is manifest at microscopic distances of 10,000-100,000 A or more. An hypothesis which seems to have some probability is that of Friedrich-Freksa (1940), according to which dipole moments develop all along the chromonema in points where basic proteins with their positive charges alternate with negatively charged thymonucleotides. The addition of 10^6 dipole moments, each of the order of magnitude of 10^{-18} u.e.s.cm. gives for two chromonemata a force of attraction of about 10^{-12} dynes, capable of attracting chromosomes at distances of 1 μ. This distance could increase if the number of dipoles is greater than 10^6. The attraction between two homologs should be more intense than between non-homologs owing to the fact that the distribution of the dipoles along the chromonemata is the same in the former.

This hypothesis rests upon the assumption, which seems probable, that a great number of dipoles appear in the chromonemata and also on the admission of a kind of dorso-ventrality of the chromosomes, with positive charges on one side and negative charges on the other. This last assumption has not been explained by Friedrich-Freksa but it would perhaps be possible to derive it from the initially double nature of the chromonemata at the beginning of meiosis (Fig. 5).

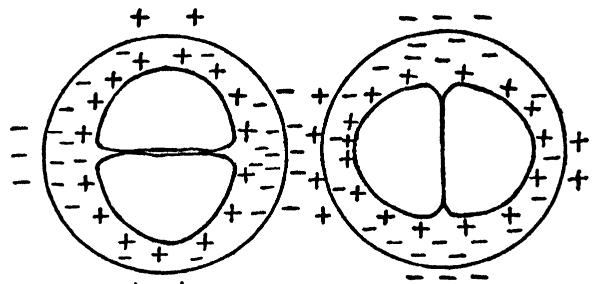

FIG. 5. A possible explanation of the distribution of + and — charges on each side of the leptotene chromosomes. The almost split chromonema has + charges, while the uniform layer of nucleic acid is negatively charged.

There are other difficulties inherent in this theory, for instance, the kind of repulsion which is manifest in salivary chromosomes when two segments are not strictly homologous, while the theory postulates that between such regions a weak attraction should prevail (if the fusion of salivary chromosomes is the result of the same forces as those acting in meiosis). This difficulty is also inherent in other hypotheses, such as that of Fabergé (1942), and could perhaps be overcome by postulating material connections between homologous chromomeres in the prophasic nucleus of meiosis and in Dipteran mitotic and salivary chromosomes. Such connections could not be rigid, as is obvious from the complicated movements taking place from leptotene to zygotene, but it is possible to postulate that the fibrils are flexible, extending at the sides of the chromomeres (Fig. 6). The fibrils could be formed by long chain molecules oriented in the nucleoplasm

in relation to the chromomeres and possibly also in relation to the interchromomeres (another kind of fibrils in the latter case). Suggestive in this respect is the kind of hairs found in lampbrush chromosomes of oocytes, which as said above are often no more than rod-shaped particles of the nucleoplasm oriented in relation to the chromonemata, as well as the less conspicuous hairs of diffuse diplotene spermatocytes. If such oriented flexible fibrils do exist, then two chromomeres should pair only when these indentations fit into one another. Of course, this hypothesis is as yet merely speculative

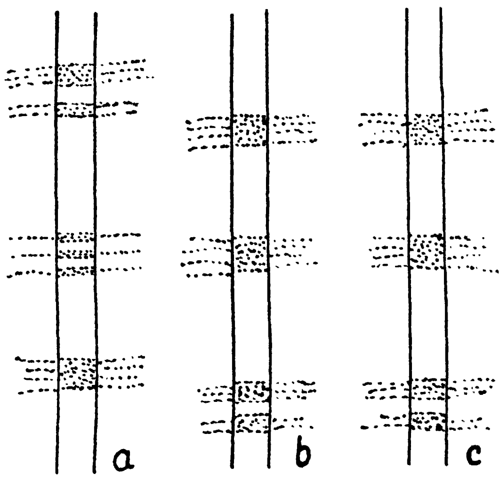

FIG. 6. Hypothesis of the flexible fibrils at the sides of the chromomeres in leptotene chromosomes. The fibrils correspond in the homologous *b* and *c*, but not between these and the heterologous *a*.

and its merit may be only to provide a basis for observations of what happens in the nucleoplasm during meiosis, observations which have been totally neglected.

If it be permissible to develop this hypothesis, we should say that the long chain molecules of the postulated fibrils must be the nucleoproteins being synthesized in the nucleoplasm at the contact points with the chromonemata. Nucleotides, probably of ribose nature, and relatively simple polypeptids rich in arginine would come from the cytoplasm and would be transformed and elaborated in the nucleoplasm; that is, the ribonucleotides would be transformed into thymonucleotides and the polypeptides into basic proteins, these two kinds of compounds combining into nucleoproteins primarily of a fibrous nature and secondarily as prophase progresses, aggregated in a nucleoprotein gel (peripheric nucleoproteins). The forces of pairing would be no more than an extension of polymerization forces which tend to draw the fibrils between homologous chromomeres, while after a certain charge of nucleoproteins is reached, gelation sets in and the chromosomes can no longer pair; on the contrary, they then become distinct and cohesion forces act in the opposite sense, a kind of repulsion then appearing. The strict specificity of the homologous pairing could be explained on this hypothesis by supposing that the chromomeres are composed of sub-units and that each chromomere has a definite pattern which would be reflected in the disposition of the fibrils.

Whatever the hypothesis adopted, meiotic pairing appears as a consequence of a slow deposition of nucleoproteins in the meiotic prophase, when compared to the prophase of ordinary mitosis in which the chromosomes are heavily charged with nucleoproteins from the beginning.

The Role of other Components in Mitosis and Meiosis

The possible role of components other than nucleoproteins will now be briefly considered. The nucleolus is visibly engaged in active metabolic changes during prolonged meiotic prophases, in connection with the elaboration of cytoplasm and reserves. Cells in active growth always have large nucleoli, partly because the volume of the nucleolus increases more or less proportionally to the growth of the cell. On the other hand, there is no doubt that the nucleolus also takes part in the process of growth, at least in oocytes, probably by elaborating ribonucleotides and basic polypeptides from materials supplied by the cytoplasm through the nucleoplasm, these compounds being afterwards transported to the cytoplasm (Serra and Queiroz Lopes, 1945). The nucleolus seems to effect partial syntheses of nucleic acid and basic proteins.

The role played in mitosis and meiosis by the nucleoplasm must also be important, but as yet few data are available on this subject. The possible role

LEGENDS FOR PLATE I (Serra) (see opposite page)

FIG. 7. Chironomus salivary gland chromosomes colored by a Feulgen reaction. The leftmost small chromosome carries the nucleolus, indicated by dots. × 480.

FIG. 8. *Chironomus Thummi* first chromosome, arginine reaction. × 1240.

FIG. 9. Idem, arginine reaction, III chromosome. Heterochromatic region marked by the arrow. The chromosome has decomposed into fibrils by pressure. × 1350.

FIG. 10. Idem, arginine reaction, IV chromosome with the nucleolus which shows a very intense arginine reaction. × 1400. (Figs. 8, 9 and 10 from Serra and Queiroz Lopes, 1944, Chromosoma, 2:576-591.)

FIG. 11. Chironomus salivary nucleus colored by a tyrosine reaction. × 1000.

FIG. 12. Salivary chromosomes of Chironomus treated by nucleases and colored by a Feulgen reaction. Only the heterochromatic regions have still a relatively great amount of thymonucleic acid.

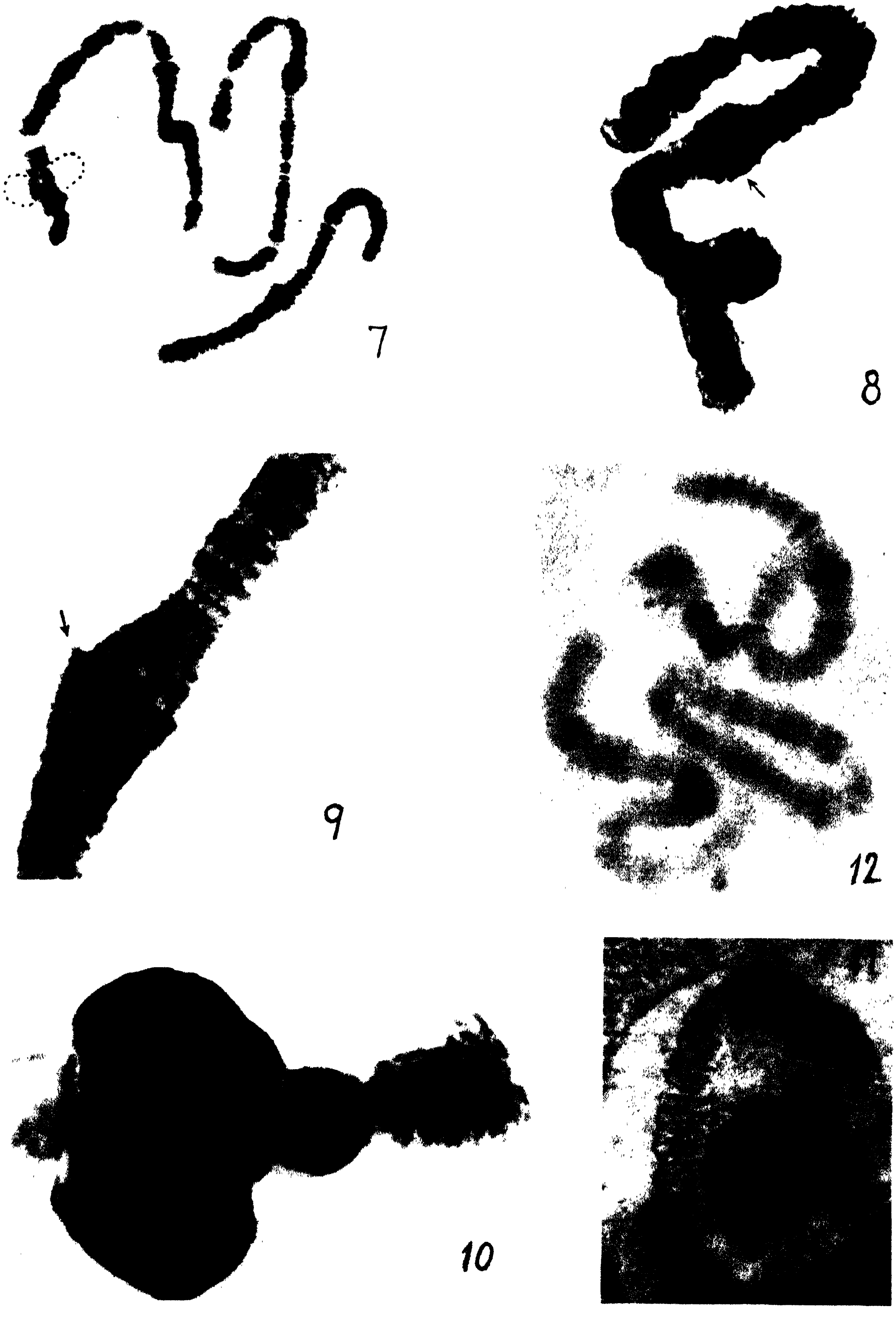

PLATE I (Serra) (see legends on opposite page)

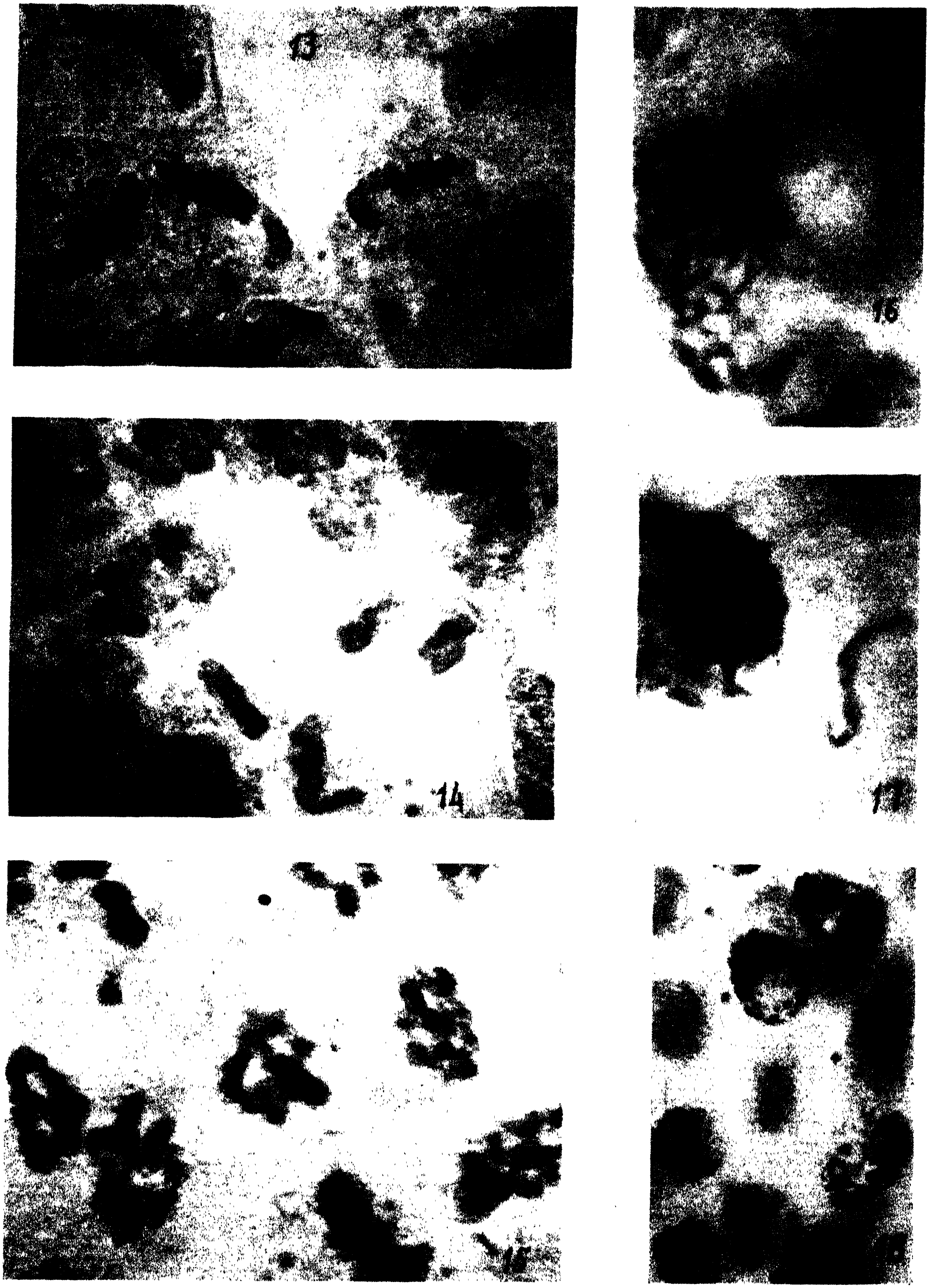

PLATE II (Serra) (see legends on opposite page)

of proteins or peptides rich in SH groups (general discussion and references in Brachet, 1944) has been widely discussed. When these groups belong to proteins their increase probably means only a more active metabolism of these compounds, since it is known that SH groups may appear when polypeptide chains distend.

An indication of the role of other components is given by the observation that chromonemata completely deprived (or with a quantity less than the tests used can reveal) of peripheric nucleoproteins are also visible in the diplotene stage of oocytes. Data on lipid color reactions, to which we have referred above, point out that very probably the lipids play a decisive role in delimiting these chromonemata from the nucleoplasm. It is also very probable that chromonemata always appear in connection with a state of unbalance of proteins and lipids in the nucleoplasm, in view of the fact that lipids are important in the structure of cell membranes. It seems to us, however, that we could not subscribe to the opinion of Monné (1945) who attributes to lipids the most important role in mitosis and meiosis, almost neglecting other components. It is seen, for instance, that it is not necessary to fix cells by means of lipid solvents in order to dissolve the karyolymph and to observe the chromonemata. Formol by itself can do the same as lipid-solvent fixatives and it is known that chromosomes may be observed *in vivo*. In salivary chromosomes, for instance, the visibility or invisibility of chromosomes depends upon the state of hydration and other properties of the nucleoplasmic colloids. It seems very probable that lipids play an important role in maintaining the structures of the cytoplasm and especially the phosphatids with their power of absorbing water. It seems, therefore, that the role played by the nucleoplasmic lipids will prove in future work to be important for the explanation of several phenomena, and especially the appearance or nonappearance of structures according to the state of balance of the lipo-proteins (Serra, 1943).

General Discussion

As the interpretations of the facts referred were discussed in some detail, the following general discussion will be chiefly devoted to comparing our interpretations with those of other workers and to an integration of our results with data of a quantitative order. The composition of chromonemata and chromosomes has been approached directly by qualitative cytochemical tests and indirectly by quantitative analysis of complexes extracted from the tissues. Although these latter methods have the great advantage of being quantitative, it is always difficult to decide if the methods of extraction are sufficiently selective in isolating only certain morphological elements of the cell. On the other hand, data obtained on isolated nuclei of glandular tissues may not apply, and possibly do not even correspond in the main, to nuclei engaged in active mitosis or in meiosis. It would be possible, however, to isolate nuclei in certain stages of mitosis in sufficient amounts for an analysis with refined micromethods.

On isolated thymus nuclei, Mayer and Gulick (1942) have found about 32% of nucleic acid, on the basis of the phosphorus content (nuclei probably free from phosphatids), and 36% histone. Another protein fraction was isolated by isoelectric precipitation at pH 5.8-6.15 and by extraction with 5% NaCl, amounting to 20% of the total N and containing approximately 0.6% S. These sulphur containing proteins are one of three things: decomposition products of histones with other proteins, a special protein, or more probably a protein mixture from the nucleoplasm, which is shown to have SH groups (the proteins extracted have their sulphur in another form, but this may be due to the isolation).

The experiments of Mirsky and Pollister (1943, 1946) performed on isolated nuclei and whole tissues gave different results, as was to be expected from the diversity of the methods employed. By extraction with 1 M NaCl of isolated nuclei or of tissues previously treated with physiological saline, which dissolves ribonucleoproteins, they have been able to prepare a nucleoprotein complex which they named chromosin, formed of thymonucleic acid, histone and other proteins. According to the evidence presented, chromosins are little or not at all contaminated with cytoplasmic components, but it is possible perhaps that nucleoplasmic and nucleolar proteins may be extracted jointly. The nucleic acid is exclusively of the desoxyribose type (sensitivity of the method, 4-5% of ribose) and amounts to

LEGENDS FOR PLATE II (Serra) (see opposite page)

FIGS. 13 and 14. Spermatocytes in the I meiosis of the grasshopper *Odontura glabricauda* Charp. Arginine reaction. In Fig. 14 the chromosomes are agglutinated (beginning of pycnosis) corresponding exactly to the image given by a color reaction for thymonucleic acid and demonstrating that basic proteins are also in the outermost layers of the chromosomes. × 1500.

FIG. 15. Idem. Chromosomes treated with carbonate buffer of pH 9.8 for 1 hour. Feulgen coloration. × 2000.

FIG. 16. *Allium cepa* root tip meristem. Fixed for 2 hours in acetic-alcohol (1: 3) and extracted for 2 hours with 1 M NaCl. Prophasic nucleus. × 2900.

FIG. 17. Idem same treatments, telophase nucleus. The chromonema detached from this nucleus (seen at the right) is double and has a chromomeric like structure. × 2900.

FIG. 18. Idem, same treatments as in Fig. 16. Contrast between 4 telophase nuclei and the other prophase and interphase nuclei. × 1500.

about 40%, with a variation of more or less 4%. Proteins form the remaining 60% and consist of histone and another protein fraction which is perhaps a mixture of several proteins, or decomposition products of proteins. The amount of histone is about 44% of the total chromosin and the other protein makes 16% of the complex (or 73 and 27% histone and non-histone proteins respectively, of the total protein fraction).

Formerly, on the basis of absorption spectra, and perhaps also from the chromonemata. (The "pellet" referred to by Mirsky and Pollister may be formed by remains of the chromonemata, but a part of these may have dissolved.)

Taking into account the direct qualitative histochemical data and the indirect quantitative data, we arrive at the composition of the nucleus and especially of the chromosomes presented in Table 1, which is no more than a guess at this complex question. The many doubts expressed in the table clearly

TABLE 1

(In this table, + indicates the presence of a substance in unknown amounts, (?) indicates doubt and 0 indicates absence.)

	Composition	Thymonucleic acid	Ribonucleic acid	Histones	Other proteins	Lipids
CHROMOSOME						
CHROMONEMATA amount variable from 10 (?) to 100% according to the matrix present	Non-basic proteins alone (?) or: Non-basic proteins alternating with nucleoproteins composed of basic proteins and nucleic acid (thymo- or ribo-) (?)	(?)	(?)	(?)	+ 100% (?)	0
MATRIX variable according to the phase of mitosis and meiosis, from 10 (?) to 90% (?)	Nucleoproteins about 90–95% (?)	35–45%	0 (?)	45–35%*	0 (?)	0
	Other components from the nucleoplasm (proteins, lipids, about 5–10% (?))	0	+ (?)	0 or +	+	+ (?)
NUCLEOLI	Proteins, ribonucleic acid, lipids	0	+ (20–30%) (?)	40–60%	+ (20–30%) (?)	+
NUCLEOPLASM	Proteins, lipids, perhaps nucleic acids	+ (transitory)	+	++ (especially in old cells	++	++

* Substituted by protamines in certain cases.

with certain assumptions about the total amount of proteins presented, Caspersson (1940) calculated that metaphase chromosomes would have about 50% nucleic acid and 50% histone. The absorption spectra upon which the distinction between basic and non-basic proteins was based have since been shown to be inconclusive when working with isolated nucleoproteins (Mirsky and Pollister, 1943, 1946) but nevertheless the general conclusions of Caspersson are not contradictory to the general conclusions drawn from histochemical data, although it is certain that metaphase chromosomes must have non-basic proteins also. The absorption spectra of metaphase chromosomes are not suitable for analysis of their proteins, due to a high content of nucleic acid.

The chromosins very probably come from chromatic threads and chromatin. From what we have said about qualitative tests and the composition of chromonemata and matrix, it seems that the nucleoproteins were extracted chiefly from the matrix but also in part from the nucleoplasm and nucleolus and show how incompletely informed we are about the basic question of the composition of the nucleus. Without more complete knowledge on this point, nuclear physiology will have no sound foundations. We hope that from this Symposium and from future work stimulated by the discussions, a more complete picture will result in a short time.

Let us now turn to the discussion of the dynamic aspects of the role played in nuclear division by nucleoproteins and nucleic acids. The functions we have attributed to nucleoproteins in mitosis are:

1. The individualization of chromosomes; and at the time of greater concentration, the individualization of two half-chromonemata from a chromonema whose proteins were already reduplicated in preceding periods of synthesis;

2. The helicoidal coiling of the chromonemata and the contraction of the chromosome by gelation of the matrix. At the end of this process the gelation reaches the chromonemata themselves.

The role of the nucleoproteins and especially of thymonucleic acid in the individualization and the

separation of the chromosomes from the nucleoplasm is obvious in nuclei of the thread type, but in other nuclei the appearance or non-appearance of chromonemata is probably connected with a state of unbalance of the nuclear lipids and especially of the phosphatids, which modifies the state of hydration and therefore the refringence and microscopic visibility of the protein structures. Less obvious is the function of the nucleoproteins in dividing an already reduplicated chromonema into two halves. On this point there is only indirect evidence, which in part is a corollary of the role played in individualizing the chromosome as a whole and in part derives from cytological observations of what happens during anaphase and telophase in suitable material. It seems probable that peptization of a part of the matrix in anaphase and telophase is the decisive factor in individualizing or separating two bundles of protein structures.

This role in separating the reduplicated structures is different from the functions attributed by several authors to nucleic acid during the synthesis of the principal cell components. A decision upon this important point depends upon the results of analyses of pure chromonemata, about which there are two alternatives: 1) they consist of proteins without nucleic acids; and 2) they are formed of alternate regions of histone nucleoproteins and of non-basic proteins. If hypothesis 2 should be valid, it would still be possible that the nucleic acid was thymonucleic or else ribonucleic. The actual data do not permit a final decision on this point. It seems premature, therefore, to conclude that the gene is a nucleoprotein, although there are several indications in favor of such a view. We have previously accepted this view but personally we would not be much surprised if the evidence took a different turn, in favor of a simply protein composition of the chromonemata and therefore of the gene. Whatever the outcome of this question, the role of the nucleoproteins during mitosis and meiosis probably has nothing to do with the reproduction of the basic structures of the chromonemata, which must be effected during the interkineses. When present in great amounts on the chromonemata and especially in a gelated state, the nucleoproteins probably hinder the metabolic changes of the chromonemata with the nucleoplasm. During division of the nucleus the functions of the nucleoproteins are rather of protection than of multiplication. A parallel hypothesis for the cytoplasm, alternative to that generally accepted, is that ribonucleic acid would play a role in cytoplasm similar to that of thymonucleic acid in the nucleus, in individualizing granules after they have been synthesized and not in taking part directly in the synthesis. Of course, at the present it is not possible to resolve these alternatives, although the hypothesis of the active role of the nucleoproteins in cellular syntheses seems to be more probable.

Concerning the role of nucleoproteins in maintaining the helical coiling and in contracting the chromosomes, of the two components, thymonucleic acid seems to be the principal one. The fibrous nature of the nucleoproteins isolated from nuclei derives principally from their thymonucleic acid (Mirsky and Pollister, 1946). It is probably the polymerization of thymonucleic acid that sets in the process of gelation characteristic of prophase. Simple modifications in the state of balance of the lipids and proteins, and therefore of water and electrolytes, in the nucleoplasm may induce a dissociation of the nucleoprotein complexes to their components and this should correspond to a peptization of the matrix. *In vitro* the nucleoproteins may be dissociated or undissociated according to the concentration of NaCl (Mirsky and Pollister, 1946). It is possible, therefore, that the balance of the nucleoplasm in electrolytes, lipids and water, together with a system of depolymerases and other enzymes, could induce the changes involved in the deposition and the dissolution of the peripheric nucleoproteins, respectively in prophase and telophase. It is suggestive that almost immediately after the chromosomes come to lie in the cytoplasm, when the nuclear membrane disintegrates, their matrix begins to be less condensed, probably by a partial dissociation of the nucleoprotein complex. The nucleoproteins of the innermost layers are more difficult to remove by extraction and also by digestion with nucleases (their nucleic acid), which suggests that a part of the nucleoproteins may be firmly bound to the chromonemata; it is this part of the nucleoproteins which persists through the interkineses and brings about the reappearance of the chromatic threads at the end of resting stage in actively dividing tissues.

Our description of the prophasic shortening of the chromosomes is very different from the scheme postulated by Caspersson (1941), according to which during prophase the interchromomeres disintegrate and the metaphase chromosomes are composed almost exclusively of the chromomeres. In telophase the converse would happen, the interchromomeres being synthesized by the adjacent chromomeres or genes. We have repeatedly criticized this hypothesis (Serra, 1942, 1946a, 1947) which has no foundation in morphological observations, and corresponds only to the chemical composition found by absorption spectra for metaphase and salivary chromosomes, the latter being taken as belonging to typical interphase nuclei. Experimentally it has been demonstrated that metaphase chromosomes can be extended without rupture to many times their length. A great stretching of a given region of a metaphase or anaphase chromosome in smears, generally of zones with less nucleoproteins like secondary constrictions, is a common observation to many cytologists. Moreover, by treatments with solvents of the nucleoproteins it is possible to reverse the shortening of the chromosomes

and to obtain a kind of prophase or interphase nucleus (Wada and others). The otherwise interesting data of Caspersson are not conclusive in this respect since by his method he cannot analyse with detail the composition, and much less the structure, of metaphase chromosomes. It can be safely concluded that a disintegration of interchromomeres, if it occur at all, could only be partial and of little importance in ordinary mitosis and meiosis. The great shortening of the chromosomes is achieved by spiralization at a microscopic level and a submicroscopic folding at a lower level.

That the proteins of the chromosomes are always submicroscopically folded (at molecular or supramolecular levels) is demonstrated by the fact that extended chromonemata, for instance of salivary chromosomes, can easily be stretched to several times their original length without rupture (Buck, 1942), probably by a mechanism of unfolding of polypeptide chains with rupture of weak bonds (passage from a more or less globular to a more fibrous state). If it were postulated that in salivary chromosomes intercalary growth has taken place between the bands, it might also be assumed that the chromomeres had also undergone intercalary growth because the bands of salivary chromosomes are obviously equal to several times the size (the length) of the corresponding meiotic chromomeres; indeed, the length of only a few bands of a salivary chromosome would almost equal the length of a whole meiotic Dipteran chromosome. The interpretation of this point which seems better to fit the known facts is that chromonemata reproduce their like by synthesizing a certain quantity of polypeptidic chains similar to their own; when a sufficient number of these chains (about 10,000-40,000 for a chromosome just visible at the microscope) is synthesized, division begins and two chromonemata are individualized during the stages with a heavy charge of peripheric nucleoproteins.

This is apposition growth. Intercalary growth, in the sense of the appearance of new molecules, seems not to occur and chromosomes increase in length by despiralization and unfolding of submicroscopic foldings, including an unfolding of a semi-globular to an almost completely fibrous (with parallel polypetidic chains) structure, according to the physiology of the cell and the presence or absence of peripheric nucleoproteins. Intercalation of chromomeres and chromosome regions seems to be possible only by alterations in the chromosome structure, by duplications and translocations (the converse, the loss, being effected by deficiencies and deletions), with a possible diversification of the chromomeres in their new places. According to this view, the chromosome is a fibrous unit composed of sub-units, its intercalary growth being possible only by increasing the number of these sub-units and not by introduction of outside molecules.

We must now refer to heterochromatin, since in recent work a great role has been attributed to it in the growth and multiplication of the cell (Caspersson, 1941; Caspersson and Santesson, 1942; Hydén, 1943). As these questions are dealt with especially in another contribution to this Symposium, only a few words will be said here. Chiefly from theoretical points of view, it has been claimed that heterochromatin is richer than euchromatin in histones (Caspersson, 1940) and that ribonucleic acid is present there in greater concentrations than in euchromatin (Brachet, 1944). In Chironomus salivary chromosomes the regions next to the centromere, which are believed to be heterochromatic, give a more intense arginine reaction but this is easily explained by the greater condensation of these regions, since they are markedly thicker than the rest of the chromosome. It is not possible to observe in Chironomus and Drosophila salivary chromosomes any difference in the color reaction of the heterochromatin by a Feulgen reaction; even the structurally diffuse chromocenter of Drosophila gives a very intense reaction for thymonucleic acid.

The amount of heterochromatin has an effect upon the nucleolus composition (Caspersson and Schultz, 1940) and size (Fernandes and Serra, 1944) but this does not imply that the nucleolus is a special elaboration of heterochromatin, since there are well developed nucleoli in species without or with little heterochromatin. This relation is probably an indirect one, derived from a slower disintegration of the nucleoproteins in heterochromatic regions. All facts taken together point to the concept that heterochromatic regions or chromosomes are different from euchromatic ones only by retention of their peripheric nucleoproteins and especially the part of them closely bound to the chromonemata. This is related to the non-functioning of these regions from a genetical point of view (Serra, in press). The heterochromatic condition may be permanent or transitory and has not the great role in cell physiology which has been attributed to it by some authors, since there are many species with little or no heterochromatin. The heterochromatization of a given region is probably a result of the relative inertness of that region from the genetic point of view and in this respect an excess of heterochromatin represents a charge for the cell, since heterochromatic regions are generally slower in dividing and in pairing and many heterochromatic chromosomes have a tendency to be lost. Heterochromatic regions are maintained by a balance between their usefulness in mechanisms of sex determination, in rendering inert certain duplicated or unbalanced fractions of the genotype, and in making stable certain special zones of the chromosomes like terminal regions and those adjacent to the centromere in many species, on one side—and on the other their detrimental properties upon the

mechanisms of division and pairing. Natural selection acts upon the net result of this balance and maintains or rejects heterochromatic chromosomes.

Finally we end this discussion by considering the special role played by nucleoproteins in meiosis. Whatever the mechanism of pairing, it seems that the special balance and distribution of nucleoproteins in leptotene is the determining agent in inducing homologous pairing. Whether dipole moments are responsible for the attraction, or flexible fibrils extend between homologous chromomeres, the forces of pairing are related to the slow deposition of thymonucleic acid and basic proteins. If the hypothesis of the flexible fibrils should be adopted, a mechanism of zipper action could be supposed to act after an intimate pairing between two homologous chromomeres was attained and long distance colloidal forces (polymerization of nucleoproteins and especially of nucleic acid) and not short distance molecular forces, should be responsible for meiotic pairing.

Recently Darlington and LaCour (1946) have referred to observations of nucleic acid aggregations not connected with chromonemata, in nuclei at the beginning of meiosis. This corresponds more or less to the diffuse type of resting nucleus characteristic of premeiotic animal spermatocytes, but by carefully observing these nuclei it seemed to us that similar aggregations were remains of chromomeres, all stages of transition between minute points and what seemed ordinary chromomeres being apparent. Whatever their significance, these observations show that nucleic acid, and without doubt basic proteins also, are only slowly being deposited on the chromonemata and that the onset of meiosis, as compared with ordinary mitosis, is characterized by a relative lack of nucleoproteins in the nucleus.

Summary

The composition of the nucleus and especially of the chromosomes, as revealed by histochemical tests, has been dealt with in some detail. Nucleoproteins whose chief components are thymonucleic acid and basic proteins play an important role in mitosis in individualizing, dividing and contracting the chromosomes, which are formed of chromonemata and peripheric nucleoproteins. In meiosis, besides playing also these roles, the special distribution and the balance of nucleoproteins between the cytoplasm and the nucleus are the principal agents responsible for meiotic pairing. The role of nucleoproteins and their components in the reduplication of nuclear and cytoplasmic structures has been discussed in connection with the nature of the gene. The nature of heterochromatin has been briefly considered.

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SOME BASIC PROBLEMS IN THE RELATION OF NUCLEIC ACID TURNOVER TO PROTEIN SYNTHESIS

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INTRODUCTION

In recent years much attention has been focussed on nucleic acids as controlling factors in protein synthesis. This is owing to the existence of evidence from which may be deduced certain correlations between chemical findings on the one hand and observation of biological function on the other. Thus, for example, it is supposed that genes control ultimately all cellular functions, including protein synthesis (see, for example, Beadle and Tatum, 1941). From the close association of genes with nucleoproteins it is natural to assume that nucleic acids may be involved in protein synthesis. Such an assumption can be supported by the fact that nucleic acids are always found associated with self-duplicating units (*e.g.*, genes, viruses, plastids, transforming principles of pneumococci and coli). It is tempting to hope that this association of nucleic acids with the capacity for protein synthesis is not entirely fortuitous.

Some considerations not at variance with this notion can be provided from modern biochemical researches which emphasize the importance of phosphate bonds for biosyntheses. Phosphorylated compounds have been shown to act as precursors in several biosyntheses. A specific example is the synthesis of glycogen (Cori, Swanson, and Cori, 1945; see also Lipmann, 1941). The energy content of the organic phosphate linkage is supposed to be an important feature of such reactions. The concept of the "energy-rich" phosphate bond removes to some extent the mystery shrouding the nature of energy coupling between synthetic reactions and catabolic processes. In particular, there is implied the conversion of endergonic synthetic steps into spontaneous reactions because the necessary energy is supplied in the molecular structure of one or more of the reactants. Simultaneously the specific point of reaction is assured by placement of the energy-rich linkage. It is reasonable to suggest an extension of this concept in elucidation of biosyntheses in general. It appears plausible to assume that phosphate-containing proteins (*e.g.*, nucleoproteins) may act as directing elements in the synthesis of cellular proteins.

Experimental evidence which may be cited in support of the crucial role of nucleic acids is extensive. It is not necessary to enter here into a prolonged discussion of these experimental results since they will be detailed in other papers of this Symposium. However there are certain aspects of the nature of the evidence which may be examined briefly.

One of the early and basic findings in this field was contributed by Brachet (1933) who showed that changes in nucleic acid content paralleled embryonic development. In these and subsequent studies (Brachet, this Symposium) analytical chemical methods were employed to ascertain the fate of nucleic acids during embryogenesis. Two different classes of embryos could be distinguished on the basis of behavior of the nucleic acids. In one type no increase in purine nitrogen occurred during a period in which ribose was being converted to desoxyribose nucleic acid. In the other type a net increase in the purine content was observed concomitant with synthesis of both ribo- and desoxyribonucleic acids. Subsequently Caspersson and his collaborators (1940; see also Hydén, 1943, for an extensive bibliography) undertook extensive studies seeking to establish a connection between protein synthesis and nucleic acid metabolism. The methods employed depended for the most part on the characteristic absorption of pyrimidine rings in the ultraviolet and as such yielded data which could not be interpreted unequivocally in terms of nucleic acids. However, a partial confirmation for such interpretations has been provided by the work of Norberg (1943) who used a cytochemical test for nucleic acid phosphorus.

In any case there appeared to be a striking parallelism between protein-synthesizing activity and nucleotide content of cytoplasm. Particularly noteworthy in this connection was work (Caspersson, Landström-Hydén and Aquilonius, 1941) which elaborated this relation in protein producing gland cells and in protein metabolism of nerve cells under various conditions (Hydén, 1943). Caspersson and Schultz (1938) found that the cytoplasm of rapidly dividing yeast cells contained a large amount of nucleotide as compared with non-dividing cells which were simply fermenting substrate (sugar). Most recently, Malmgren and Hydén (1947) have provided evidence for a parallelism between nucleotide content and division activity of bacterial suspensions.

While the experiments cited show that protein synthesis or modification is associated with marked changes in nucleotide synthesis, none can be interpreted to exhibit a rigorous causal relation be-

tween the two processes. Furthermore, procedures which are concerned only with quantitative determination of compounds can yield information only on *net* changes. There exists the possibility that a particular class of compounds is actively involved in metabolism without undergoing any net gain or loss in absolute amount. Hence, it is desirable to determine turnover as well as gross content. Data of this nature is readily obtained with the aid of isotopic tracers. Several investigators have used radioactive phosphorus (P^{32}) in attempts to obtain information on the relative metabolic activity of the nucleic acids in various tissues. Hahn and Hevesy (1940) found a slow but appreciable turnover of nucleic acid phosphorus in all rabbit tissues examined. In the case of normal rabbit liver the data indicated that only 33% of the nucleic acid was replaced in 50 days. However, all other forms of organic phosphorus were renewed completely in the course of a few days. Marshak (1941) in experiments with isolated liver nuclei reported a rapid turnover of phosphorus in resting liver. The apparent discrepancy in these results may be explained, perhaps, by the data of Brues, Tracy, and Cohn (1944). These authors remarked that when the estimate of turnover was made on isolated nucleic acids, the values obtained agreed with those of Hahn and Hevesy. However, when the turnover of "total" protein phosphorus (trichloracetic acid precipitate freed of phospholipid) was measured, the turnover found was much greater. There appeared to be cellular phosphate associated with protein which was not nucleic acid phosphate. This phosphate was involved in a rapid turnover. Brues, Tracy, and Cohn suggested that the data cited by Marshak related to this non-nucleic acid fraction. All workers agreed that regenerating liver exhibited a much higher turnover in nucleic acid phosphate than resting liver, a result to be expected.

The experiments performed with tracer phosphorus have not provided direct and unambiguous evidence relating to the role of nucleic acids as controlling factors in protein synthesis. The fact, for example, that desoxyribosenucleic acid incorporates phosphate extensively only during cell growth is not convincing evidence for its importance in synthetic activity. The same fact would be true for any phosphate compound which is formed or duplicated only during cell division.

An attempt to obviate this difficulty has been made in some recent experiments (Spiegelman and Kamen, 1946) in which division was minimized by limiting the amount of nitrogen available for synthesis. In these experiments the flow of phosphate from the "nucleoprotein" fraction was studied under various conditions. This was done by growing yeast cells in P^{32}-labeled medium and then allowing such cells to ferment glucose in the presence of unlabeled phosphate. Under such conditions the acid soluble phosphate was lowered in specific P^{32} content more rapidly than the acid insoluble phosphate because the acid soluble phosphate equilibrated more rapidly with the exogenous unlabeled phosphate. With this differential in specific P^{32} content established, it was possible to use the cells to follow the flow in phosphate between the two fractions. It was found that the addition of exogenous nitrogen (N) as ammonium sulphate led to a marked drop in P^{32} content of the acid insoluble fraction. In the absence of N assimilation, however, no such decrease was observed. Further, it was shown that the presence of any agents (*e.g.*, sodium azide or 2-4 dinitrophenol) which prevented the assimilation of nitrogen or the formation of new enzymes inhibited at the same time the transfer of phosphate from the acid-soluble fraction, assumed to be mainly "nucleoprotein" phosphate.

The validity of conclusions derived from these results concerning the relation of protein synthesis and nucleic acid metabolism depend on two factors: (1) the composition of the "nucleoprotein" fraction; and (2) the comparative turnover of other fractions during the same experimental period. The importance of nucleic acid can only be established if it forms the dominant portion both with respect to percentage composition and rapidity of turnover. It is requisite, therefore, that data on the behavior of other phosphate fractions be included, if the analysis is to be considered adequate. It is the purpose of this paper to describe experiments and data obtained therefrom which permit comparison of phosphate turnover in the nucleic acids with that in other phosphate compounds in the cell.

Experimental Procedures

A. *Mechanism of Entry of Phosphate*

It is customary to compare the P^{32} content of the various fractions with that of the internal orthophosphate of the cell. This practice stems from the assumption that the internal orthophosphate is a common pool from which all the various organic phosphates eventually derive their phosphate. The mechanism of entrance of exogenous phosphate is usually represented in the following way:

$$\text{(I)} \qquad IP_o \xrightarrow{(1)} IP_i \xrightarrow{(2)} OP_i$$

where IP_o is the external orthophosphate, IP_i is the internal orthophosphate, and OP_i is the internal organic phosphate. Aside from the experiments of Furchgott and Shorr (1943) there exists scanty experimental support for this scheme. These authors showed that at 2° C., the P^{32} content (specific activity) of the IP_i fraction was higher than that of any organic P examined. (The cellular material in this case was mammalian muscle slices.) From this result it could be concluded that diffusion of phosphate as inorganic orthophosphate into the cell could occur. However it was also found that the entry of phosphate at 37° C. was 5-10 times more rapid than at 2° C. This argued against the supposition that only a simple diffusion mechanism

was operative. It seemed desirable to investigate this matter further, particularly with respect to whether the internal orthophosphate afforded the best basis for comparison of cellular phosphate fractions. Some previous results on phosphate exchange under various conditions appeared to offer an opportunity for securing pertinent data. Under normal conditions of glucose fermentation in the presence of exogenous phosphate, the phosphate content of yeast cells increases. Hotchkiss (1944) reported briefly on some aerobic experiments which indicated that sodium azide can prevent phosphate uptake by yeast cells respiring in the presence of glucose. Since only net changes were being observed, it was not possible to decide whether this effect was due to inhibition of phosphate penetration or whether factors such as esterification, storage and utilization were also involved.

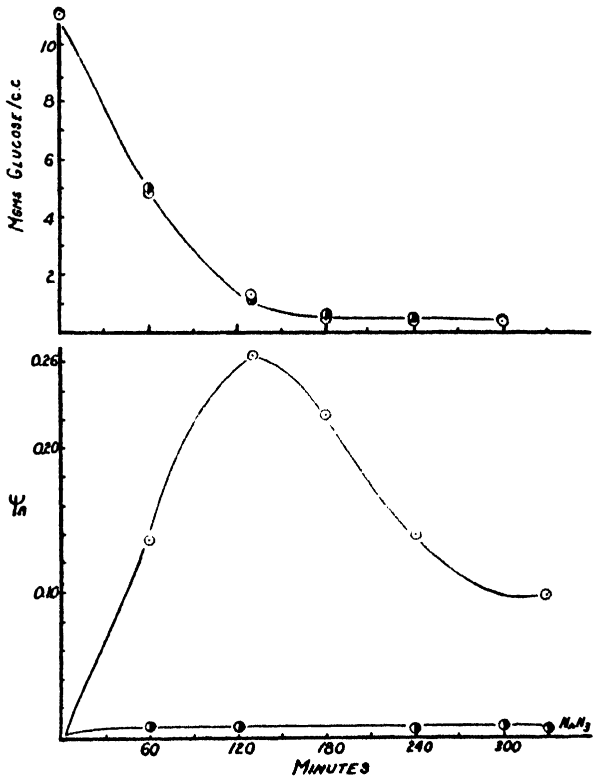

FIG. 1. Effect of Sodium Azide on Phosphate Exchange in Fermenting Yeast Cells. In the upper curves the concentration of glucose remaining in the fermentation medium is plotted as a function of time. In the bottom curves the ratio of the specific P^{32} content of the cellular phosphate to the specific activity of the exogenous phosphate (Ψ_R) is plotted for the same interval of time. The open circles indicate results obtained in the absence of NaN_3; the shaded circles exhibit results obtained in the presence of NaN_3 (2.5×10^{-3} M)

A more detailed examination of this phenomenon was undertaken using labeled phosphate. The general results obtained are illustrated in Fig. 1. A suspension of yeast cells was allowed to ferment anaerobically a given amount of glucose in the presence of labeled phosphate, with and without sodium azide (2.5×10^{-3} M) in the medium. At intervals samples were removed, the cells washed free of contaminating exogenous labeled phosphate and assayed for both P^{32} and P^{31} content. Simultaneously the concentration of glucose in the fermentation medium was determined. The upper curves of Fig. 1 show the results of the reducing sugar determinations. It is seen that, with the concentration of azide used, no interference with glucose fermentation occurred. In the lower curves Ψ_R, the ratio of specific activity (counts/min/γ P) of the internal phosphate to that of the exogenous phosphate, is plotted as a function of time. Ψ_R multiplied by 100 is the percent equilibration of the internal phosphate with the exogenous phosphate.

TABLE 1. PHOSPHATE CONTENT* IN YEAST CELLS AFTER 90-MINUTE FERMENTATION IN PHOSPHATE-FREE MEDIUM WITH AND WITHOUT AZIDE (2.5×10^{-3} M)

Fraction	Zero	Control	Azide
Acid Soluble Orthophosphate	95	51	85
Organic phosphate	145	121	124
Acid Insoluble	215	250	206
Medium	0	40	50
Total	455	462	465

* All figures are amounts of phosphate in gammas ($\gamma = 10^{-6}$ gms.).

From Fig. 1 it is clear that azide affected most strikingly the ability of the cell to exchange its phosphate with that contained in its environment. If the mechanism pictured in the reaction diagram (I) were operative one might postulate that azide prevented either step (1), step (2), or both. If only step (2) were being blocked by azide then, since about 10% of the internal phosphate is orthophosphate, one should observe about 10% exchange. Since, however, only 2% exchange relative to the control was found it appeared that azide also interfered with step (1).

However, it should be remembered that azide interferes with all the internal synthetic processes of the cell. Since these can take place in the absence of exogenous phosphate it is clear that processes other than simple penetration of phosphate into the cell must be involved. This could be shown more directly with respect to phosphate metabolism by the following experiment. Yeast cells were allowed to ferment glucose anaerobically in the presence and absence of azide (2.5×10^{-3} M) and without exogenous phosphate. At the end of 90 minutes, the cells were collected by centrifugation. Analyses were made of the inorganic and organic

phosphate in the acid soluble fraction together with the phosphorus content of the acid insoluble fraction and medium. The results are shown in Table 1, in which are given the amounts of phosphorus found in the various fractions. Comparison of the values obtained after a fermentation period of 90 minutes with those obtained at zero time revealed several effects due to the azide. In the control cells, 47% of the orthophosphate disappeared; whereas in the presence of azide only 11% was lost from this fraction. The acid-insoluble phosphate of the control increased 16%; in the presence of azide this fraction lost 4%. Both suspensions lost about the same amount of phosphate to the medium. It was clear from these results that azide most probably interfered with the internal transfer of phosphate from the inorganic fraction to the organic fraction.

It seemed profitable to entertain the idea that orthophospate did not enter the cells as such, but rather that phosphate was taken up as an organic phosphate compound, the formation of which depended on cellular metabolism. Diagrammatically,

$$\text{(II)} \qquad IP_o \rightarrow OP_i \leftrightarrows IP_i$$

Cellular orthophosphate could be supposed to result mainly from the breakdown of organic phosphate compounds. As will be seen, this hypothesis afforded a simple explanation of how azide, by interfering with the esterification of phosphate, could at the same time inhibit exchange between internal and external orthophosphate.

The ability of azide to interfere with this exchange was tested further by examining the overall equilibration of phosphate. Cells were allowed to ferment glucose one hour anaerobically in labeled phosphate medium with and without azide. Subsequently the cells were harvested, washed, and fractionated with cold 5% trichloracetic acid (TCA) into acid-soluble and acid-insoluble components. The inorganic phosphate was separated from the acid-soluble fraction by precipitation with magnesia mixture at alkaline pH. The fractions so obtained were assayed for both P^{32} and P^{31} content. The results are recorded in Table 2. The inorganic fraction of the control suspension attained an activity corresponding to 20% of that of the exogenous phosphate. The inorganic fraction in the azide treated suspension exchanged only to the extent of 1.2%. There appeared to be a somewhat greater effect of the azide on the exchange of organic phosphate. In any case it was clear that azide exerted a strong inhibitory effect on the equilibration of internal and external orthophosphate.

TABLE 2. THE EFFECT OF AZIDE ON PHOSPHATE TURNOVER OF YEAST FERMENTING GLUCOSE

	Acid Soluble				Acid Insoluble	
	Inorganic		Organic		Residue	
Treatment	% Exchange	% of Control	% Exchange	% of Control	% Exchange	% of Control
Control	20	—	9.2	—	9.1	—
Azide (2.5×10^{-3} M)	1.2	6	0.1	1	0.1	1

These values were attained after one hour of anaerobic fermentation of glucose.

These experiments indicated that the problem of entry of extracellular phosphate could not be separated from the process whereby orthophosphate was incorporated into the organic fraction. Any experimental conditions which interfered with the latter process also blocked the uptake of extracellular phosphate.

On this basis it would be expected that azide could somehow influence those steps in the metabolic cycle which are concerned with phosphate esterification. Under anaerobic conditions, one of these steps is the coupled oxidation of phosphoglyceraldehyde to phosphoglycerate. An attempt was made to see whether the presence of azide altered the glycolytic mechanism at this level. This particular oxidation is controlled by the enzyme triosephosphate oxidase, which is relatively sensitive to iodoacetic acid (IAA). Hence, an examination was made of the sensitivity of glucose fermentation to IAA in the presence and absence of NaN_3 at various concentrations. A concentration of IAA was chosen (2.5×10^{-4} M) which resulted in complete inhibition of fermentation within 10 minutes. However, in the presence of 5×10^{-3} M NaN_3, IAA had no effect on the fermentation rate for 50 minutes. Almost two hours elapsed before the rate fell to zero. It was found that higher concentrations of NaN_3 protected to a greater extent. However, when azide concentrations were tried which in themselves began to exert inhibitory effects (60% and higher) on the fermentation rates, the protective action of the azide against IAA inhibitions was lost. It may be noted further that the effect of the azide on the glycolytic mechanism was reasonably specific, since the presence of azide did not affect the ability of fluoride to block fermentation.

These results are consistent with the view that azide is acting directly on one of the primary reactions which leads to the formation of organic phosphate. If the quantitatively important pathway for the entrance of phosphate is via esterification to an organic compound, it is not surprising that azide

can depress phosphate exchange between internal and external orthophosphate.

There is one further type of experiment which can provide information on the mechanism of internal and external orthophosphate interaction. If the primary mechanism is one of direct exchange between extra- and intracellular orthophosphates, the expected kinetics would be those derivable from the

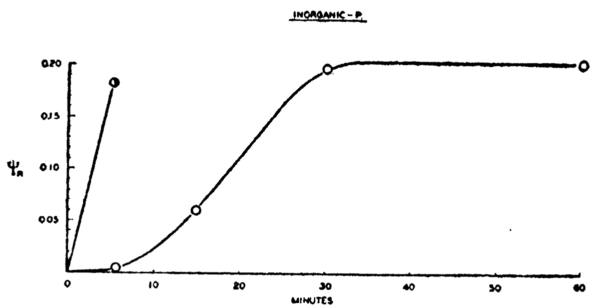

FIG. 2. Effect of Pre-incubation with Glucose on P Uptake in Fermenting Yeast Cells. Ordinate has same significance as in Fig. 1. Shaded circle is uptake datum after prior incubation with glucose for three minutes. Open circles give uptake data without prior incubation.

laws of diffusion. On this basis a plot of specific activity of the internal orthophosphate against time would result in a curve concave to the time axis which would approach asymptotically a value corresponding to complete equilibration with the external orthophosphate. When such an experiment was performed, however, there was obtained a result given by the open circles of Fig. 2. The curve was S-shaped and had a lag period, which under the conditions of this experiment was of 5 minutes' duration. The existence of this lag period would indicate that the cells ferment the sugar for a short period and build up certain compounds before they can begin to accept the exogenous phosphate. Strong confirmation for this view was afforded by the following experiment. Cells were allowed to ferment sugar for 3 minutes in the absence of exogenous P, after which labeled P was introduced. At the end of 5 minutes a sample was removed and the activity of the internal orthophosphate determined. The half-shaded circle in Fig. 2 gives the result. It is clear that the three minute preincubation in the absence of phosphate created a condition which permitted immediate and rapid partial exchange between internal and external orthophosphates.

Another aspect of the curve in Fig. 2 has significant implications for the problem of a meaningful comparison of turnover rate in the various cellular fractions. Since the ordinates represent relative specific activities, it is clear that the orthophosphate fraction in the cell was *not* equilibrating with exogenous inorganic phosphate. The apparent saturation activity was only 20% that of the external phosphate.

There are several explanations one might offer for this phenomenon.

(1) The method (cold TCA extraction) used for preparing the orthophosphate fraction actually creates a large part of it by hydrolysis of very labile organic phosphate esters.

(2) Several kinds of orthophosphate exist. Some are actively involved in the phosphate cycle. Others, because of intracellular geometrical factors, cannot enter appreciably into the phosphate cycle.

It is impossible at the present time to distinguish between these possibilities. In any case, it is clear that serious difficulties are raised in the interpretation of comparative data on turnover. Thus, it is by no means certain which part of the orthophosphate fraction should be used as a basis of comparison, the slowly or rapidly exchanging components. Further, this situation renders uncertain the extent to which the orthophosphate fraction is a common pool upon which the organic fractions can draw.

In view of this situation, it appeared most reasonable to use as a basis for comparison the external orthophosphate which possessed at least the virtue that such phosphate was controllable in composition and time-independent. All relative specific activities recorded in this paper have therefore been calculated by reference to the specific P^{32} content of the external orthophosphate.

B. *Fractionation of Yeast*

1. General Procedure

The method of chemical fractionation used was elaborated from a number of procedures available in the literature, based primarily on studies of phosphate composition of mammalian tissues (Umbreit, Burris and Stauffer, 1945). It must be emphasized that in yeast cells the distribution of phosphate was sufficiently unlike that observed in animal muscle or liver so that much uncertainty arose regarding the purity of fractions obtained. The procedure finally adopted, while admittedly arbitrary, represented a reasonable combination of existing methods appropriate for the organisms used in these studies. The difficulties created by the use of this procedure will be made evident in the following discussion.

The fractionation scheme used is shown diagramatically in Fig. 3. Yeast cells (*S. cerevisiae*, 48-hour broth cultures) were incubated under anaerobic conditions for stated periods in phthalate buffer (pH = 4.5) containing 6% glucose and 1/60 M, P^{32} labeled, inorganic orthophosphate (with or without a nitrogenous source). The cells then were washed several times with distilled water, and suspended in 5% trichloracetic acid for one hour at 3° C. The supernatant (S_1) contained all "acid-soluble" phosphate. This fraction should have contained all inorganic orthophosphate and organic phosphate compounds, particularly carbohydrate fermentation intermediates and coenzymes.

The inorganic orthophosphate found undoubtedly arose in part from labile organic phosphate. This supernate was treated with magnesia mixture at alkaline pH to precipitate the inorganic orthophosphate (fraction P_2). This left "organic" phosphate in solution (S_2). The precipitate (P_2) was found to contain not only inorganic orthophosphate but also a considerable quantity of labile phosphate, which could be converted to orthophosphate by hydrolysis for seven minutes with boiling 1 N HCl. (Any phosphate compounds behaving in this fashion will hereafter be termed "7-minute hydrolyzable" or "7-minute phosphate," symbolized Δ_7.) This hydrolyzable phosphate was characterized as inorganic metaphosphate polymer by precipitation with Ba^{++} at pH = 2.5 (P_3), in accordance with the observations of Wiame (1946; see also Schmidt, Hecht and Thannhauser, 1947). The earlier work of Macfarlane (1946; see also Mann, 1944) also should be cited in this connection. The solution (S_3) remaining after separation of metaphosphate contained the "true" inorganic orthophosphate.

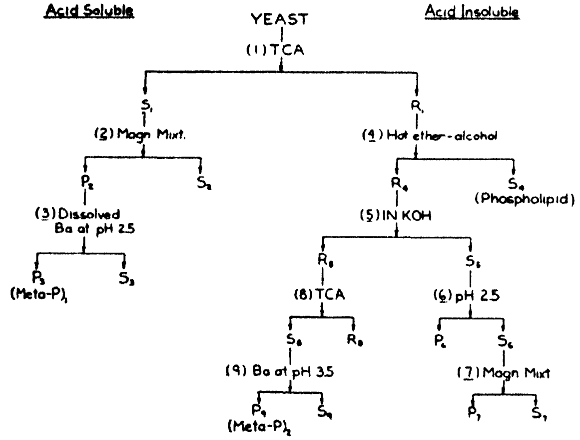

FIG. 3. General Fractionation Scheme for Yeast Phosphate.

The residue (R_1) resulting from the acid extraction was suspended once in cold alcohol, centrifuged, and extracted for 3 minutes with hot ether-alcohol mixture (three parts ether to one part alcohol) at 68-70° C. The hot ether-alcohol extraction was repeated twice so that all phospholipid and other fatty material was brought into solution (S_4). The residue (R_4) was subjected to 1·N KOH at 37° C. for twenty-four hours, as in the extraction procedure of Schmidt and Thannhauser (1945). This hydrolyzes ribose nucleic acids to nucleotides. Desoxyribosenucleic acids remain virtually intact, while phosphoproteins are split to protein and inorganic orthophosphate. Thus there was obtained fraction (R_5), the "KOH residue," and fraction (S_5) containing all phosphate present originally as nucleic acid phosphate and phosphoprotein. This phosphate now appeared as ribose nucleotides, desoxyribose nucleic acid, and orthophosphate. Solution (S_5) was adjusted to pH = 2.5 with trichloracetic acid and HCl according to the method of Schmidt and Thannhauser (1945). There resulted a precipitate (P_6) which presumably contained only desoxyribose nucleic acid phosphate. The solution (S_6) was brought to alkaline pH and treated with magnesia mixture to precipitate orthophosphate (P_7) which should have represented phosphate present initially as phosphoprotein. The supernate (S_7) thus should have contained only ribose nucleotide phosphate.

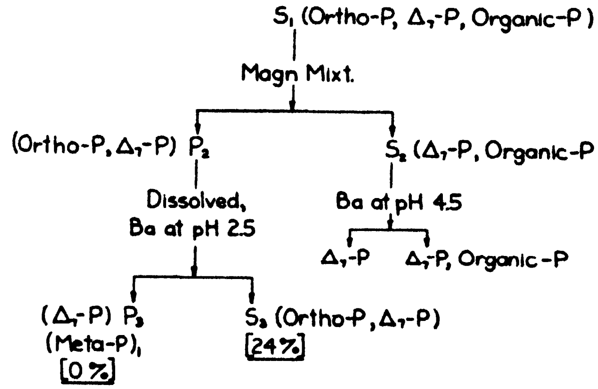

FIG. 4. Fractionation of Acid Soluble Phosphate. Numbers in brackets refer to percent equilibration with exogenous labeled phosphate.

The KOH residue (R_5) was subjected to extraction with 10% trichloracetic acid for ninety minutes at room temperature. Acid-soluble phosphate present in this residue was found subsequently to be extractable equally well with distilled water. However, acid extraction was employed to ensure removal of all acid-soluble phosphate. The solution (S_8) was treated with Ba^{++} at pH = 3.5 (acetate buffer), which resulted in the appearance of a precipitate (P_9) and supernate (S_9). The barium precipitation was used to characterize, in a preliminary way, the phosphate (R_5) which unexpectedly had resisted solution in KOH.

2. Detailed Fractionation of Acid-Soluble Phosphate

In Fig. 4 is shown the complete fractionation procedure for acid-soluble phosphate. It has been remarked that, in the precipitate (P_2) resulting from treatment with alkaline magnesia mixture, there was included a large amount of seven-minute phosphate. It seemed plausible from a study of the literature (Wiame, 1946; Macfarlane, 1936; Mann, 1944) to suppose that such a hydrolyzable fraction was inorganic metaphosphate. It was shown that this labile fraction was not pyrophosphate by its failure to precipitate when Cd^{++} was added at pH = 4.5. Furthermore, addition of a known quantity of pyrophosphate resulted in a precipitate

which contained no phosphate in excess of that added. To test the supposition that the labile fraction was in actuality metaphosphate, Ba^{++} was added to the solution obtained by treatment of the magnesia precipitate (P_2), and the pH was brought to 2.5 (acetate buffer). The characteristic metaphosphate precipitate appeared. A confirmatory test with toluidine blue gave the expected metachromatic color (Wiame, 1946; Schmidt, Hecht and Thannhauser, 1947). All phosphate in this precipitate was seven-minute hydrolyzable. It appeared on the basis of these tests that the labile phosphate appearing with inorganic phosphate could be ascribed entirely to metaphosphate. Other workers (Wiame, 1946; Macfarlane, 1936) had demonstrated that the barium-precipitable material obtained in this fashion exhibited a percentage phosphorus and barium content identical with the theoretical value expected for metaphosphate. The precipitation was carried out at pH = 2.5, rather than at the higher pH (4.5) recommended, to insure a high purity for the isolated metaphosphate. This was requisite because it was desired to ascertain its specific labeled (P^{32}) content; and contamination with other acid-soluble phosphate, particularly the inorganic orthophosphate of high specific (P^{32}) content, had to be minimized. About 50% of the total metaphosphate originally precipitated with the magnesia mixture (P_2) was recovered in the barium precipitation at pH = 2.5.

The supernate (S_3), containing about 50% of the total metaphosphate and all of the inorganic orthophosphate from the original precipitate (P_2), could be used for estimation of the specific P^{32} content of the orthophosphate. A correction for the contaminating metaphosphate was applied after assay of the purified metaphosphate obtained as described above.

The supernate (S_2) remaining after the initial magnesia precipitation should have contained only organic phosphate. However, an unexpectedly large percentage of this phosphate was found to be seven-minute hydrolyzable. On adjusting this solution to pH = 4.5 (acetate buffer) and adding Ba^{++}, a precipitate formed containing 50% of the total labile (seven-minute hydrolyzable) phosphate in solution (S_2). The magnesia precipitation procedure was known to result in only partial precipitation of metaphosphate. Hence it seemed likely that the excess labile phosphate remaining in solution (S_2) was metaphosphate. The relatively high pH (4.5) employed was dictated by the necessity for removing all such contaminating metaphosphate so that, in the ensuing assay of P^{32} content, a reliable value for the specific P^{32} content of the organic acid-soluble phosphate could be obtained. At pH = 4.5 a relatively minor fraction of the acid-soluble organic phosphate was precipitated, while all metaphosphate could be demonstrated to have been removed. The barium precipitate obtained from the organic acid-soluble phosphate contained only seven-minute phosphate, and appeared to be almost entirely metaphosphate.

3. Detailed Fractionation of Fat-free Acid Insoluble Residue (R_4)

Treatment of the fat-free acid-insoluble residue with 1 N KOH, followed by fractionation with acid and subsequent precipitation employing magnesia mixture in alkaline medium (Fig. 5) should have achieved clean separation of the ribose nucleotide, desoxyribose nucleic acid, and phosphoprotein phosphates, assuming these to be the only kinds

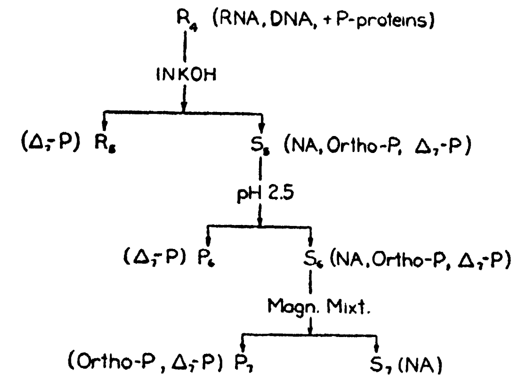

FIG. 5. Fractionation of Fat-Free Acid Insoluble Phosphate (Schmidt).

of phosphate present. Examination of the fractions obtained disclosed the failure to achieve complete separation. It was necessary to revise preliminary interpretations based on the assumption of adequate purity of samples.

Considering only solution (S_5) and its derived fractions, it became apparent that in the "desoxy" fraction (P_6) there was a large amount of seven-minute phosphate contaminated with much proteinaceous matter. This contamination, which constituted nearly 80% of the total phosphate present in (P_6), did not appear to be metaphosphate, pyrophosphate, or nucleotide phosphate; for a precipitate could be obtained neither at acid pH with Ba^{++} or Hg^{++} nor with magnesia mixture in alkaline medium. The only characteristics established for this unknown phosphate were lability toward acid hydrolysis (seven minutes at 100° C. in 1 N HCl) and solubility in 5% trichloracetic acid. The true desoxyribose nucleic acid of the cells comprised about 20% of the phosphate in this fraction, and in relative percentage of cell phosphate appeared to check closely the value expected from estimates in the literature.

The possibility that the labile phosphate could have resulted from the action of KOH on desoxyribose nucleic acid was considered. It was found that in a known sample of desoxyribose nucleic acid, the phosphate was labilized to some extent by a prior incubation with 1 N KOH for 24 hours at

room temperature, the increase in seven-minute phosphate over that present initially being 6%. In 3 hours with 1 N KOH at 100° C. the desoxyphosphate was labilized to the extent of 13%. It is clear that only about 1% out of the original 80% labile phosphate in this fraction could be ascribed to the KOH treatment. Estimation of the specific P^{32} content in desoxyribose nucleic acid required a complete separation of the contaminant phosphate from the desoxyphosphate.[1]

Another complication appeared when solution (S_6) was fractionated to isolate so-called inorganic

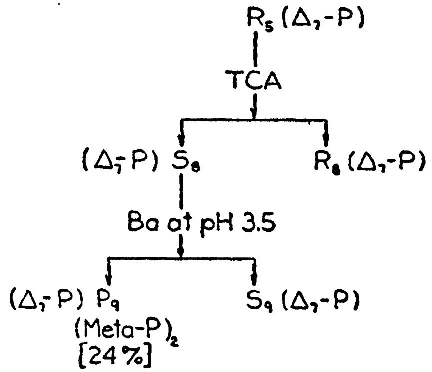

FIG. 6. Fractionation of KOH Residue. Number in brackets has same significance as in Fig. 4.

orthophosphate presumed to originate from yeast phosphoprotein. The precipitate (P_7) obtained with alkaline magnesia mixture was found to be contaminated with the apparently ubiquitous labile phosphate. This labile phosphate was found to comprise 67% of the total phosphate in precipitate (P_7). Because of its characteristic precipitation with Cd^{++} at pH = 4.5 and Zn^{++} at pH = 6.5, this contaminant appeared to be accounted for as pyrophosphate. The supernate (S_7) behaved in proper fashion; all the phosphate could be accounted for completely as ribose nucleotide phosphate by spectrophotometric analysis.

4. Detailed Fractionation of KOH-Residue (R_5)

As remarked previously the existence of an insoluble residue after KOH extraction was most unexpected. Under the conditions of these experiments, a surprisingly high percentage of phosphate was found in this fraction. The residue was analyzed first (Fig. 6) for labile phosphate, in view of the experiences with other fractions. Seven-minute hydrolysis in boiling 1 N HCl decomposed practically all the residue phosphate in (R_5) to inorganic orthophosphate. This observation recalled the experience of Macfarlane (1936), who found that some metaphosphate in yeast resisted prolonged extraction with cold trichloracetic acid. This type of metaphosphate was found by Macfarlane to be easily extractable immediately upon neutralization of the suspension containing the acid insoluble components.

The residue (R_5) was suspended in 10% trichloracetic acid and extracted at room temperature for ninety minutes. The supernate (S_8) was separated from the residue (R_8) by centrifugation, and Ba^{++} added at pH = 3.5 (acetate buffer) to test for metaphosphate. A characteristic metaphosphate precipitate was obtained. The phosphate in

TABLE 3. PHOSPHATE DISTRIBUTION IN THE YEAST CELL

Nature of Fraction	Type of Phosphate	% of Total P
Acid Soluble	Orthophosphate	9
	Metaphosphate	19
	Organic phosphate	10
Acid Insoluble	Phospholipid	3
	Phosphoprotein	2
	Pyrophosphate	4
	Nucleic Acid	22
	Metaphosphate	17
	Unidentified (1)	11
	Unidentified (2)	4

(1) Associated with acid insoluble metaphosphate.
(2) Associated with nucleic acid fraction.

this precipitate was accounted for almost entirely as seven-minute phosphate, and exhibited a typical metachromatic color with toluidine blue.[2]

Supernate (S_8) and residue (R_8) contained, in addition to some residual metaphosphate not removed by precipitation, an additional fraction (or fractions) consisting of phosphate as yet unidentified.

C. *Analytical Data*

The composition of yeast phosphate in yeast cells (using 48-hour cultures harvested and then incubated for one hour in glucose-phosphate medium) is shown in Table 3. These data are in good agreement, wherever comparison is possible, with results given by Macfarlane (1936). When the cultures were incubated in the presence of sufficient ammonium sulfate to supply nitrogen for assimilatory activity but insufficient for appreciable growth (cell division), the composition of the various fractions changed in the manner shown in Table 4. It is seen that in these "resting" cultures the major change occurred in the so-called acid-insoluble metaphos-

[1] A procedure which accomplishes this separation has recently been elaborated and the analysis of the P^{32} content of the purified desoxyphosphate will be reported elsewhere.

[2] These tests were conducted in collaboration with Dr. J. M. Wiame, who also noted recently the appearance of an acid-insoluble metaphosphate fraction in yeast.

phate fraction, in which an increase of 50% in gross phosphate content is noted. Other fractions appeared unchanged, or even showed a decrease, the most appreciable decrease being found in the nucleic acid fraction. The significance of these data is best discussed in connection with the presentation of data on the specific P^{32} content of the various fractions.

D. *General Pattern of Phosphate Turnover*

Details of the determination of specific P^{32} content for the diverse fractions need not be considered in this report. The results of the radioactivity assays are exhibited in Table 5. The phosphate turnover is referred in all cases to the P^{32} content of the exogenous labeled phosphate. Thus, a figure of 9.9% as given for orthophosphate in column one means that the specific activity of this fraction is 9.9% that of the specific activity in the exogenous phosphate.

It will be noted that the level of equilibration rises markedly in the presence of an exogenous nitrogen source. The largest increase in turnover, relative to the control without the nitrogen source, is seen in the nucleic acid fraction. The increase is nearly nine-fold (880%) and is much higher than can be accounted for by the decrease in gross phosphate content of this fraction as given in Table 4 (about 16%). The relative turnover in other fractions is also greatly enhanced, that in the acid-insoluble metaphosphate ranking with that in the nucleic acid phosphate.

A most striking result is the complete lack of turnover in the inorganic acid-soluble metaphosphate with or without exogenous nitrogen. The complete absence of radioactive phosphate in this fraction is the more remarkable in that it is isolated from a fraction with the highest specific activity, namely inorganic orthophosphate. It appears that the metaphosphate in yeast is not only heterogeneous with respect to acid solubility, but is physiologically differentiated into at least two types. One type is associated apparently with protein (or nucleic acid) and enters into rapid metabolic turnover, while the other type (present presumably as "free" metaphosphate) is not involved in turnover. This finding is of some importance in connection with the general validity of tracer experiments which purport to demonstrate relative metabolic activity on the basis of measurements of specific tracer activity. This point will be considered in more detail in the discussion.

It is important to know the variation of specific activity in the diverse fractions with time. The time course of turnover typical of yeast phosphates is exemplified in Fig. 7. There is shown the increase in specific activity of various fractions relative to the specific activity of exogenous labeled phosphate during anaerobic fermentation of glucose without an external source of nitrogen. The most important

TABLE 4. AMOUNT OF PHOSPHATE IN VARIOUS FRACTIONS OF YEAST

The figures are in mgms., based on the analysis of 10-gm. (wet weight) samples of yeast after three hours under anaerobic conditions in the presence of 6% glucose and M/60 KH_2PO_4. The tracer phosphate was added at the end of the first hour. Samples with nitrogen were provided with $(NH_4)_2SO_4$ corresponding in amount to 50% of the protein nitrogen content of the yeast.

Nature of Fraction	(1) Without nitrogen	(2) With nitrogen	(2)−(1)
Acid Soluble			
Orthophosphate	3.2	4.0	+0.8
Metaphosphate*	7.0	6.3	−0.7
Organic*	4.3	3.8	−0.5
Acid Insoluble			
Phospholipid	1.2	1.1	−0.1
Phosphoprotein & Pyrophosphate	3.2	2.5	−0.7
Ribonucleic Acid	9.1	7.5	−1.6
KOH Residue**	10.5	16.0	+5.5

* These fractions have been corrected for the metaphosphate remaining in the organic fraction after magnesia precipitation.

** 60% of this fraction consists of acid-insoluble metaphosphate whose specific activity is higher than that of the crude fraction.

TABLE 5. TURNOVER VALUES FOR THE VARIOUS PHOSPHATE FRACTIONS WITH NITROGEN AND WITHOUT NITROGEN

The figures represent per cent equilibration with external labelled phosphate attained at the end of three hours of anaerobic incubation in 6% glucose and M/60 KH_2PO_4. The tracer phosphate was added at the end of the first hour.

Nature of Fraction	(1) Without Nitrogen	(2) With Nitrogen	$\frac{(2)}{(1)}$
Acid Soluble			
Orthophosphate	9.9	39.4	4.0
Metaphosphate*	0.0 (<0.03)	0.0 (<0.03)	—
Organic*	7.2	27.1	3.8
Acid Insoluble			
Phospholipid	3.3	14.3	4.3
Phosphoprotein & Pyrophosphate	2.5	12.3	4.9
Ribonucleic Acid	0.9	7.1	7.8
KOH Residue**	6.2	33.9	5.6
Metaphosphate	5.0***	39.8	8.0***

* Corrected for metaphosphate not precipitated with magnesia mixture.

** Contains 60% of its phosphate as acid-insoluble metaphosphate.

*** Calculated with assumption that distribution of activity between metaphosphate and other phosphate in KOH residue is same as with nitrogen.

feature of these curves is the failure of any fraction to reach equilibrium with exogenous phosphate during the time of the experiment. The saturation values for relative specific activities are reached relatively rapidly, at least in the case of inorganic orthophosphate. Other fractions appear to approach the same saturation value (about 20% of the theoretical value for complete equilibration of labeled phosphate) with rates characteristic of each. All fractions seem to turn over phosphate less rapidly than the inorganic orthophosphate fraction. In only one case (KOH residue) is the same maximum value reached as that exhibited by the inorganic orthophosphate. In Fig. 8 there are displayed complete time curves for the same experiment from which the data were taken at the 120 minute interval for Table 3. Fig. 8 shows clearly the relation in turnover between the diverse phosphate fractions as influenced by assimilatory activity in the presence of external nitrogen. As noted previously, the nucleic acid phosphate shows the greatest relative enhancement in turnover. However, the KOH residue phosphate not only exhibits a remarkable increase in turnover, but appears to be increasing its P^{32} content at a time when all other fractions have reached saturation values. It is not known whether the levels in relative specific activity finally attained in these experiments are true equilibrium values, or whether experiments of greater duration would result eventually in the attainment of the values expected for complete equilibration with exogenous phosphate. Attempts to carry out such experiments have been defeated by autolysis of cultures maintained for inordinately long periods in phosphate buffer under anaerobic conditions. Autolysis of the cultures results, of course, in complete phosphate equilibration.

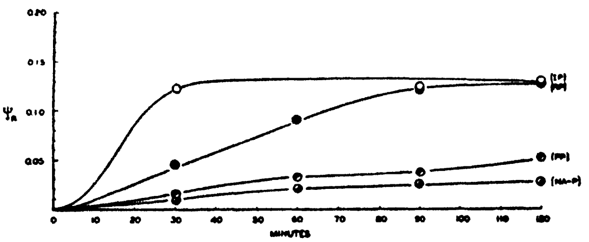

FIG. 7. Relative Specific Activity of Phosphate Fractions as Function of Time. Ordinate has same significance as in Fig. 1. Open circles = orthophosphate; solid circles = total acid insoluble phosphate; top shaded circles = "Phosphoprotein" and insoluble or pyrophosphate; bottom shaded circles = nucleic acid phosphate.

Discussion

The major purpose of the experiments described here was to obtain a set of data which would permit an evaluation of the relative importance of nucleic acid in nitrogen assimilation and protein synthesis. In devising the experiments it seemed essential to be able to compare nucleic acid activity with that of other fractions. This would avoid the justifiable criticism of any experiment which focussed attention on only one fraction. It also was important to avoid cellular multiplication, for under such conditions all fractions would be equally labeled.

In two respects the data obtained can be considered to lend some support to the importance of nucleic acid in nitrogen metabolism. First, it will be noted that in Table 4 the assimilation of nitrogen was accompanied by a drop in nucleic acid. Other than the "metaphosphate fraction," the nucleic acid was the only one to be markedly affected by the nitrogen assimilation. The fact that a fall in the nucleic acid fraction was observed might be interpreted as utilization of the nucleic acid for the formation of protein. Second, it will be noted from Table 5 that the turnover in the nucleic acid responded most markedly to the assimilation of nitrogen.

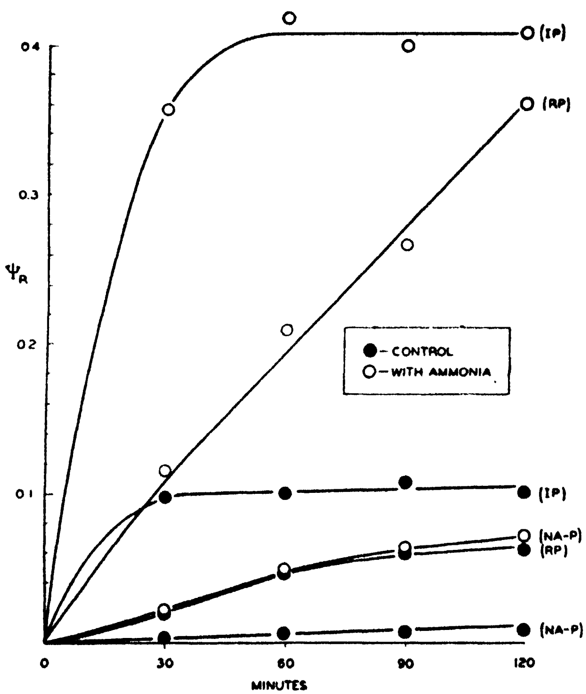

FIG. 8. Turnover of Phosphate in Fermenting Yeast Cells in Presence and Absence of Exogenous Nitrogen. Ordinate has same significance as in Fig. 1. Open circles refer to P-uptake in presence of nitrogen sufficient for assimilation but not for growth (cell division); shaded circles show uptake of control cells without exogenous nitrogen.

IP = orthophosphate
RP = KOH-residue phosphate
NAP = Ribose-nucleotide phosphate.

The forcefulness of the conclusions derivable from these findings on the behavior of the nucleic acid is certainly weakened by the fact that other

phosphate fractions also respond markedly to the onset of active nitrogen metabolism. Thus, even the phospholipid turnover increased almost four-fold during the nitrogen synthesis. Cells are highly integrated systems and it is not surprising to find that accelerated nitrogen metabolism should lead to widespread effects reflected in a diversity of cellular metabolic systems some of which may be only indirectly concerned with protein synthesis.

The existence of this situation makes it extremely difficult to obtain a set of data which could lead to any unequivocal decisions as to the importance of nucleic acid. It is quite evident from the experiments described here and the results obtained that simple comparative data are of dubious value for a satisfactory solution to the problem of protein synthesis and the role of nucleic acids in this process. At best one may be able to exhibit a quantitatively more impressive change in the nucleic acid phosphate as compared with other fractions when the cell is synthesizing protein.

One more point should be emphasized. As noted in a previous section, the finding of physiologically differentiated forms of metaphosphate forcefully illustrates the danger of using tracer techniques without due regard for possible artifacts created by the chemical methods employed. Thus, had the cells been extracted with hot rather than cold trichoracetic acid, *all* metaphosphate would have appeared lumped in one fraction on subsequent isolation and purification. The turnover value derived from the assay of such a fraction would have been merely an average of the various turnover values characteristic of the two different kinds of metaphosphate. Such an average value would give an erroneous impression of the metabolic function of metaphosphate as evidenced by turnover data.

It is difficult to quiet the suspicion that differentiation, perhaps more extensive than noted in these experiments, occurs not only with metaphosphate but also with all other yeast phosphate fractions.

In considering the general problem of protein synthesis it appears highly unlikely that only one type of phosphorylated compound (i.e., nucleic acids) can be the key to the entire mechanism. In this connection it is of interest to note the existence of polyphosphorylated (both meta and pyro) compounds associated with the protein of the cell. From the standpoint of both percentage composition and turnover during assimilation they represent one of the most active organic-phosphate fractions in the cell. It is particularly interesting to note that Macfarlane (1936) and earlier Kossel (1893), had found metaphosphate associated closely with the nucleic acid. Most recently Wiame (1947) has presented data which indicates that acid-insoluble metaphosphate may be utilized during the formation of new cellular protein. This author suggests that in view of the anhydride nature of the bonds in the metaphosphate molecule it might be used as a source of energy for synthetic purposes.

In any case, it becomes necessary to investigate the relationship of these polyphosphate-containing proteins to the nucleoproteins. It may very well be that in certain cells they form parts of the protein synthesizing mechanism.

Summary

The point of view with respect to the nucleic acid-protein synthesis problem presented in this paper may be summarized as follows. For reasons, most of which have been listed in the introductory paragraphs, it seems plausible to assume that the nucleic acids are critically important in controlling protein synthesis. The experimental evidence is as yet far from convincing. The existence of many correlations between protein synthesis and changes in nucleic acid may be of little pertinence for the evaluation of the validity of this assumption. The attempt described in the present paper to provide more critical evidence on this point has only served to raise questions as to the value of such correlative evidence. Almost equally good correlations can be established between protein formation and the metabolic activity of other phosphate fractions.

Clearly other types of experiments are needed. These experiments must be designed on the basis of some hypothesis as to the function of nucleic acids. Recently (Muller, 1947; Spiegelman and Kamen, 1946) it has been suggested that the nucleic acids serve as the agents which funnel energy into the protein synthesizing mechanism. If this is so, one might expect that the phosphorus of the nucleic acid would turn over faster than other parts of the molecule. Experiments employing both N and P tracers would yield information on this point. A comparison of such data in the presence and absence of nitrogen synthesis should be illuminating. Such experiments in which the turnover rates of different components of the same molecule are compared would avoid some of the difficulties mentioned previously as peculiar to tracer experiments.

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DISCUSSION

BRACHET: Since I was among those who first brought attention to the possible intervention of ribonucleic acid in protein synthesis, I wish to recall the fact that in the cells this acid is closely linked to a number of enzymes; in fact, these ribonucleoprotein granules contain in a specific way not only ribonucleic acid but also essential respiratory enzymes as well as ATPase. It is thus possible that the agent of protein synthesis is not ribonucleic acid alone, but the whole complex particle. Is something known concerning the presence of metaphosphoric acid containing proteins or of enzymes breaking down metaphosphoric acid in various protein fractions obtained by differential centrifugation?

SPIEGELMAN: As far as I know nothing is yet known about the distribution of metaphosphate containing proteins obtained by differential centrifugation or by any other method. There exists a powerful metaphosphatase in yeast, but its distribution is unknown.

BRUES: In the studies made by Tracy, Cohn, and myself, as described by Dr. Spiegelman, the "nucleic acid" was partially purified desoxyribose nucleic acid. In subsequent studies, we separated both nucleic acids in pure form and found that ribose nucleic acid has a much greater turnover than the desoxyribose acid. About 20% of the protein phosphorus was not removed from protein in the extraction of ribose nucleic acid, and this was discarded. Thus, we cannot be certain whether metaphosphate is present in liver, but this could probably easily be determined.

MAZIA: Dr. Spiegelman's data showing no difference in the amounts of nucleic acid in inactive and growing yeast cells are in direct contradiction to the conclusions drawn by a number of workers who used the ultraviolet absorption method. Is there some experimental condition which accounts for the discrepancy, or does Dr. Spiegelman consider the ultraviolet method to be unreliable?

SPIEGELMAN: In the first place, I do not believe that the experimental conditions are comparable. Our suspensions were not actively dividing, whereas in most instances estimations of nucleic acid content by ultraviolet were made on actively growing cells. Our comparisons were between nondividing cells which were assimilating nitrogen and those which were not. Our data indicate a decrease in NA content at the onset of nitrogen assimilation. It should be noted that our analytical estimation of nucleic acid phosphate is considerably lower than those obtained by the UV method. The data given by MacFarlane are even lower than ours. This discrepancy between the analytical and the UV results may be due to the fact that the ultraviolet absorption method does not distinguish between nucleic acid and pyrimidine containing compounds. On the other hand, a difference in experimental conditions may explain it, since wide variations in NA phosphate can be observed. Only those who possess the relatively elaborate and costly set-up required for UV determinations are in a position to check this point. All that can be said at present is that the various values obtained by different workers using analytical chemical procedures are in good agreement.

BOIVIN: The quantity of nucleic acid in bacteria varies with the age of the cultures (Boivin, A., and Vendrely, R., C. R. Soc. Biol. *137:* 432, 1943; and more recent unpublished experiments). In *B. coli* (several strains studied), in *S. aureus,* in *V. cholerae,* the value of the ratio $\frac{\text{total nucleic acid}}{\text{total protein}}$ increases by 50%, 75%, or sometimes 100% when one passes from resting bacteria (bacteria cultivated on broth during 20-24 hours at 37°) to bacteria in the lag phase or in the beginning of the logarithmic phase (resting bacteria placed during 3 to 6 hours in new broth—1000 $cm.^3$ medium for each gr. bacteria, wet weight—aerated by a slow current of sterile air). In absolute value, the ribonucleic acid increases much more than the desoxyribonucleic acid during the time when bacteria prepare for their growth (lag phase) or begin this growth. Therefore, one must conclude that in bacteria, as in animal cells, in plant cells and in yeast (Brachet, Caspersson), the ribonucleic acid participates, in some way, in the synthesis of proteins, synthesis which is linked with growth.

Our findings are in good accordance with the more recent results of Caspersson and his co-workers (Malmgren, Thorell, Bjerkelund, and Caspersson, Nord. med. Ark. *28:* 2636, 1945; Malmgren and Hydén, Nature *159:* 577, 1947; Caspersson, Symp. Soc. exp. Biol. *1:* 127, 1947, "Nucleic Acid: The relations between nucleic acid and protein synthesis") obtained on bacteria by ultraviolet technique, and with the results of Belozersky (Cold Spring Harbor Symp. Quant. Biol. *12:* 1-6, 1947). Thus from chemical analysis given in the report of Belozersky, one can calculate that the ratio $\frac{\text{total nuclei acid}}{\text{total protein}}$ is 0.39 for a 5-hour-old *coli* culture and 0.19 for *coli* 20 hours old; 0.46 for Flexner 5 hours old and 0.27 for Flexner 20 hours old. This corresponds approximately to our variations of ratio when we pass from resting to growing bacteria.

THE CHEMICAL NATURE AND FUNCTIONS OF THE COMPONENTS OF CELL NUCLEI

EDGAR STEDMAN AND ELLEN STEDMAN

About four years ago, basing our views on what we believed to be the firm foundation of the results which we had obtained in an investigation of the chemical nature of cell nuclei, we advanced a theory (Stedman and Stedman, 1943; Stedman, 1944) of the structural, and to some extent physiological, functions of the nuclear components. This theory received much adverse criticism (Callan, 1943; Barber and Callan, 1944; Caspersson, 1944), and many of the objections which have been raised to it have been recorded in a recent review (Gulland, Barker and Jordan, 1945). While, in our view, these criticisms are, as we have indicated (Stedman and Stedman, 1943, 1944), invalid, our original deductions have lost none of their force. On the contrary, they have been so reinforced by our subsequent investigations (Stedman and Stedman, 1944; Stedman, 1945) that we feel that no reasonable doubt can now remain of the essential correctness of our original conclusions. We therefore propose first to give an account of the facts which we have ascertained about the composition of cell nuclei and the nature of the nuclear components and then to point out in somewhat more detail than originally the deductions which can be drawn from them regarding the structural and physiological functions of the various components.

The Chemical Composition of Cell Nuclei

The classical investigations on the composition of cell nuclei, which were concerned almost exclusively with the heads of fish sperm and the nuclei of avian erythrocytes, have been reviewed in a recent publication (Stedman and Stedman, 1947) and need not therefore be considered here in detail; it will suffice to recall that, on the basis of the earlier work, it has long been accepted that the dry, lipoid-free heads of salmon sperm (Miescher, 1897), herring sperm (Mathews, 1897; Steudel, 1911-1913; Steudel and Peiser, 1922) and the sperm of the common white fish (Lynch, 1920) as well as the nuclei from fowls' erythrocytes (Ackermann, 1904) are to all intents and purposes composed entirely of protamine or histone nucleate, although it should be recalled that the conclusion attributed to Miescher, while based largely on his data, does not possess his authority but is a deduction made by Schmiedeberg subsequent to his death. Taken in conjunction with the discovery of a histone nucleate in the thymus gland of the calf (Lilienfeld, 1894) and of the presence of protamines and histones in the sperm of many fishes (Kossel, 1928), these results have legitimately led to the conclusion that all cell nuclei are similarly composed. Such a view has, indeed, recently been advocated by Mirsky and Pollister (1943) who have prepared nucleoproteins from various tissues (and in some instances from isolated nuclei) which they state are composed entirely of histone or protamine nucleate and are obtained in yields which represent almost the whole of the nuclear material. In two papers, however, which have just been published (Pollister and Mirsky, 1946; Mirsky and Pollister, 1946) these authors have to some extent revised their views, a point which will be discussed more fully below in relation to our own work.

Our own experiments on the composition of cell nuclei were commenced as a preliminary to a contemplated investigation into the possible differences between the chemical composition, and hence the physiological properties, of the nuclei of normal and malignant cells. Consideration of this problem convinced us that the accepted view of the chemical nature of cell nuclei could not be reconciled with their cytological and genetical properties, and it thus became necessary first to re-examine the validity of the apparently conclusive results of the earlier workers mentioned above. For this purpose it was desirable to work with nuclei, in particular with the heads of salmon and herring sperm, of the same origin as those used by these investigators. Unfortunately, these were not at the time accessible to us and only became so later at irregular intervals and usually in small amounts. We therefore turned our attention to other nuclei such as those from thymocytes, cancer cells and cod sperm. Examination of such nuclei showed that they resembled one another in containing, not two, but three main components: nucleic acid, a histone and a second protein distinct from a histone. The nucleic acid was recognized by its well known and probably unique physicochemical properties; the histone was characterized by its basic properties, by its conversion into a sulphate insoluble in aqueous alcohol, and by a positive Millon and negative tryptophane reaction; and the second protein was shown to be different from histone by its lack of basic properties and, in particular, by its giving a strong tryptophane reaction. That this latter protein was not an adventitious impurity but was an integral component of the nuclei seemed to be proved conclusively by the fact that it could be separated by a uniform procedure from three different types of

nuclei requiring widely differing techniques for their isolation. Thus, in the early stages of our work it was obtained from the heads of cod sperm, from the nuclei of fowls' erythrocytes and from the nuclei of thymocytes and tumour cells. The sperm heads were prepared by the excellent method developed by Miescher, the erythrocyte nuclei by laking the washed corpuscles and completely removing the haemoglobin by repeatedly washing the liberated nuclei with saline, while for the remaining nuclei Stoneburg's method (Stoneburg, 1939), somewhat modified and carried out in such a way as to avoid the use of enzymes, was employed. By obtaining qualitatively uniform results with different types of nuclei prepared by widely diverse methods, we not only provided ourselves with a guarantee that the tryptophane-containing protein did, in fact, originate in the nucleus, but also obtained considerable evidence that this protein could be regarded as a constant component of cell nuclei in general. Other considerations, to be discussed below, indicated, moreover, that it was not only a component but probably the most important, if not the sole, component of the chromosomes. It was therefore termed chromosomin, a name chosen according to accepted chemical terminology and meaning "the protein of the chromosomes."

In addition to carrying out the above qualitative examination of cell nuclei, we attempted to obtain analytically some measure of their content of chromosomin. This was desirable because isolation experiments by the methods which were at that time available to us, although demonstrating the presence of considerable quantities of chromosomin in all the nuclei examined, could obviously give only a very rough idea of the proportion actually present. By making the assumption, which our qualitative examination had indicated was substantially correct, that only three components, namely, nucleic acid, basic protein (histone or protamine) and chromosomin, were present in appreciable amount, it became possible roughly to assay the chromosomin content. For this purpose we determined, as Miescher had first done with salmon sperm heads, the nucleic acid and basic protein contents of the dry lipoid-free nuclei: the former by a phosphorus determination, and the latter by direct and exhaustive extraction of the protein from the nuclei by dilute mineral acid and its gravimetric estimation in the form of its characteristic sulphate. The chromosomin content could then be calculated by difference. The method is, as we have formerly pointed out (Stedman, 1944) subject to certain errors and approximations. Nevertheless, it served to demonstrate that the dry weights of many nuclei are by no means wholly accounted for by their contents of nucleic acid and basic protein, and that if, as our qualitative experiments had suggested, chromosomin was the only remaining major component it must constitute a considerable proportion of the dry-weight of the nucleus.

In carrying out these analyses we unfortunately introduced a systematic error in the values given for nucleic acid by assuming in the computation of our results that the water content of the air-dried nuclei was identical with the water content of pure, air-dried sodium nucleate; and it is conceivable that our earlier figures are subject to other experimental errors. For these and other reasons we have carried out some further analyses on certain nuclei

TABLE 1. ANALYSES OF NUCLEI FROM LYMPHOCYTES

Species	Source	Nucleic Acid Content (%)	Yield of Histone Sulphate (%)
Man (Child)	Thymus	37.0	27
Man (Child)	Thymus	36.5	23
Mouse	Spleen	35.8	28
Mouse (Strong A)	Spleen	38.0	28
Ox	Thymus	37.8	29

Note: The nuclei used in the above analyses were isolated from individual thymus glands but from mixed spleens.

in which the phosphorus determinations have been made on nuclei dried to a constant weight in a high vacuum over phosphorus pentoxide. As before, the basic protein was estimated gravimetrically as yield of sulphate (air-dry) from air-dry nuclei. The amount of sulphuric acid combined with the basic protein is, in most cases, unknown. The figures obtained cannot, therefore, be corrected for this

TABLE 2. ANALYSES OF NUCLEI FROM LIVER CELLS

Species	Nucleic Acid Content (%)	Yield of Histone sulphate (%)
Man (Child)	27.3	17
Man (Child)	27.5	17
Man (Child)	27.4	18
Ox	29.2	22
Ox	30.4	26
Mouse	28.9	22
Mouse (Strong A)	30.7	22

Note: With the exception of the mouse, the above analyses were made with nuclei isolated from individual livers.

factor and hence obviously give exaggerated values for the contents of basic protein. On the other hand, it is possible that the extraction of this component was not complete. This was certainly the case, as will be mentioned below, when protamine was involved, but we believe that only negligible amounts of histone escaped extraction. Some of the results obtained by this method are given in Tables 1 and 2. While the figures necessarily differ somewhat from those previously recorded, they are nevertheless in essential agreement with them. For example,

our earlier figures for the nuclei of thymocytes from the calf showed a yield of 21% of histone sulphate and a content of 44% of nucleic acid. The new and more accurate figures in Table 1 give values ranging from 23 to 29.5% for the histone sulphate and from 35.8 to 38% for the nucleic acid. If chromosomin is the only remaining component of these nuclei it is clear that this protein represents about 30% of their dry weight, and isolation experiments give yields agreeing approximately with this figure.

But the results in Tables 1 and 2 are of interest in another direction. The former shows that the nuclei of lymphocytes and thymocytes from three different species of mammals (man, ox, mouse) are practically identical in composition. The small variations which are observed in different preparations are no greater when they are derived from different than from the same species. Much the same agreement is to be seen in the composition of the nuclei from the liver cells of the same three species recorded in Table 2, although there is here one exception: the yields of histone from the nuclei from three different human livers are appreciably, and the contents of nucleic acid somewhat, lower than those found for the corresponding nuclei from the ox and the mouse. It is probable, although not yet certain, that this is related to the fact that the human livers were obtained from infants, there being some grounds for believing that the histone content of the nuclei from young, and hence presumably proliferating, cells is lower than that of corresponding nuclei from the adult animal. We have not, however, yet had an opportunity of making a direct comparison between nuclei of the same type obtained from young and adult animals of the same species.

In addition to demonstrating the approximate constancy in composition of the nuclei of corresponding cells from different species of mammals, the above results suggest that each nucleus possesses a normal composition which is characteristic of the type of cell in which it exists. Thus, in the mammals examined, the nuclei of lymphocytes differ from those of liver cells in their nucleic acid and histone, and hence presumably in their chromosomin, contents. The characteristic values obtained from mammals do not, however, necessarily apply to other classes of animals. Preliminary results which we have obtained with the nuclei from the salmon suggest, in fact, that in this species the nuclei of the liver cells contain a higher nucleic acid and a lower histone content than is found in mammals.

The quantitative results which we have obtained with the heads of fish sperm have shown considerable variation. Our original experiments (Stedman and Stedman, 1943) dealt with cod sperm and demonstrated that the nuclei from these cells contained a relatively low nucleic acid and high chromosomin content. We cannot doubt the essential accuracy of this result, although it is possible that our analytical figures may require some revision when circumstances permit us to repeat the work in the light of our subsequent experience with the sperm heads of other species.

Our first examination of the heads of herring sperm gave results somewhat similar to those of the cod, indicating a chromosomin content approaching 40%. With the object of checking our experimental procedure, the minced testes were, in this experiment, divided into two portions. The first was suspended in water, stirred vigorously with a mechanical stirrer, strained through muslin and the filtrate made faintly acid by the addition of a few drops of dilute acetic acid. The sperm heads were then sedimented in the centrifuge, suspended in water and again centrifuged and this process repeated until the supernatant was perfectly clear after centrifuging for 5 minutes at 1500 r.p.m. The second portion was treated in exactly the same way, except that the minced testes were stirred in a medium of 5% citric acid in place of water, this procedure rendering, of course, the subsequent acidification with acetic acid unnecessary. Both preparations were dried, and the lipoids removed, by two extractions with cold absolute alcohol and one with ether. On extraction of the dried sperm heads with 0.5 N sulphuric acid, exhaustively as we believed at the time the experiment was carried out, both preparations yielded substantially the same quantity of protamine sulphate, the figures being 21% and 20.5% respectively for the water and citric acid preparations. A phosphorus determination on the water preparation indicated a nucleic acid content of approximately 40%. These experiments were carried out in 1944 and, apart from effecting a partial separation of the chromosomin from the nucleic acid, no further examination of the material was at that time made. In view, however, of the widely different analytical results which we have recently obtained with a new specimen of herring sperm heads we have submitted these two old preparations to a re-examination. On microscopic examination both appeared to consist entirely of sperm heads without admixture with extraneous material. Both contained identical amounts of phosphorus and hence of nucleic acid, the actual figures being: water preparation, 4.09% P, 41.3% nucleic acid; citric acid preparation, 4.07% P, 41.1% nucleic acid; and both yielded 17% of their weight of protamine sulphate on extraction as exhaustively as possible with 0.1 N hydrochloric acid. The diminution in yield of protamine sulphate as compared with the earlier experiments was, of course, due to the use of 0.1 N acid in place of 0.5 N for its extraction. That this was the case was confirmed by the subsequent isolation of protamine from the nucleic acid fraction extracted from the residue. It is thus apparent that repeated extraction (up to 10 times) of dried sperm heads with 0.1 N mineral acid, which is the procedure we have generally adopted for assaying the basic protein content of nuclei, does not remove protamine quanti-

tatively. When, as in cod sperm heads or other nuclei in general, the basic protein is a histone the extraction appears to be much more complete, although we cannot exclude the possibility that traces still remain in the residue. If it is assumed that 0.5 N acid effected the complete extraction of the protamine from the above preparation of herring sperm heads, the percentage of residue, consisting mainly of chromosomin, can be calculated as follows: Of the 21% of protamine sulphate isolated, 72% consists of protamine and 28% of sulphuric acid. The sperm heads thus contain 14% of protamine. They also contain, according to the phosphorus determination, 41% of nucleic acid, these two components thus accounting for 55% of the sperm heads. Allowing 5% of the residue for systematic errors such as the incomplete extraction of protamine and the presence of inorganic material or of minute quantities of other unknown substances, we conclude that this specimen of herring sperm contained approximately 40% of chromosomin. While we have not succeeded in isolating this amount of pure chromosomin from the sperm heads we have, by fractionating the residue left after removal of the protamine, obtained, despite inevitable losses in the process, as much as 31% of material consisting of chromosomin contaminated with only a small amount of nucleic acid. The actual content of the latter has not yet been determined, but qualitative tests suggest that it is much smaller than in the preparation from another batch of herring sperm heads now to be described.

This new material was prepared from some ripe herring testes in February of this year. The sperm heads were isolated by the water method described above and appeared on microscopic examination to be completely pure. Incidentally, it should be mentioned that neither in this nor in the earlier preparation were the heads of the u-shape pictured in Fig. 121 of Wilson's classical work (Wilson, 1925); on the contrary, they resembled the sperm heads of the salmon in being flattened symmetrical bell-shaped objects with a prominent indentation at the base. On extraction with 0.1 N hydrochloric acid, exhaustively as we believed at the time, they yielded in two experiments 23% and 24% respectively of protamine sulphate. Examination of the nucleic acid extracted from the residue, however, showed that this was contaminated with protamine which had evidently not been removed during the acid extractions. We therefore submitted another sample of the same preparation to extraction with N hydrochloric acid. By this means we were able to obtain a yield of 30% of protamine sulphate, which corresponds with about 22% of protamine itself. Duplicate phosphorus determinations gave identical values of 5.85%, the nucleic acid content hence being 59.1%. Protamine nucleate thus accounts for 81% of the dry weight of the sperm heads of this preparation, leaving 19% for chromosomin and any other substances which may be present. To isolate chromosomin from sperm heads or nuclei containing such a high content of nucleic acid is a matter of considerable difficulty and certainly cannot be effected quantitatively by the methods at present available. Nevertheless, we were able to isolate it from this material in a yield of 10% of the dry weight of the sperm heads. This specimen was, it is true, contaminated, as shown by a Feulgen test, with nucleic acid. A phosphorus determination, moreover, gave a value of 0.85%. Assuming that chromosomin contains no phosphorus, an assumption which has not yet been verified, this indicates a nucleic acid content in the specimen of 8.6%.

As we are convinced that this remarkable quantitative variation in the composition of the two preparations of herring sperm heads is a real one, we have sought some explanation of the result, which we now believe is to be found in the fact that the testes which we employed were derived from two different varieties or races of herring. The material used in 1944 was obtained from fish of about 10 inches in length, such as are normally found in the North Sea and around the coasts of Britain. On the other hand, that prepared during the present year originated from much larger fish, 13 to 14 inches long, similar to those which spawn off the west coast of Norway. Such a finding offers an explanation of the results, referred to above, obtained by Mathews and Steudel in their investigations of the composition of herring sperm heads. If, as is probable, these authors used testes from the Norwegian race of herrings, the chromosomin would easily escape detection owing to the relatively high proportion of nucleic acid present in the sperm heads from this race.

Our investigations of the sperm heads from the salmon are in some respects less complete than those from the herring. It is, however, possible to say that they resemble the sperm heads derived from the Norwegian race of herrings, as we have conveniently described the larger race of this fish, very closely in composition. One experiment has already been described elsewhere (Stedman and Stedman, 1947) in which chromosomin, admittedly contaminated with a small amount of nucleic acid, has been isolated in a yield of 20% of the dry weight of the sperm heads. Further incomplete experiments have, however, shown that there are slight differences in the composition of the sperm heads obtained from different individuals. This is illustrated by the following percentages, which give the phosphorus contents, with the corresponding nucleic acid values, determined on sperm heads isolated from the testes of five different individuals: 5.34, 53.9; 5.88, 59.5; 6.02, 60.8; 6.12, 61.8; 6.22, 62.8. The nucleic acid contents thus vary round the value of 59.1%, obtained with the sperm heads from the mixed testes of the herring (Norwegian race) and our preliminary experiments indicate that the protamine content is also of the same order of magnitude. If this be provisionally assessed at 22%, there remains from 15% to 20% of the dry weight of the sperm

heads unaccounted for as protamine nucleate, and our isolation experiments show that the greater part of this residue must consist of chromosomin. Detailed figures are, however, not yet available.

Nature and Distribution of the Nuclear Components

The qualitative and quantitative examination, dealt with in the preceding section, of the nuclei from lymphocytes, thymocytes, liver cells and certain fish spermatozoa has shown that these nuclei are composed of three major components: nucleic acid, basic protein (histone or protamine) and chromosomin. Work on the nuclei from other cells is not yet complete, but the qualitative examination of the nuclei from bird and fish erythrocytes and from many tumour cells of both human and animal origin has shown that these, without exception, also contain the same components. It therefore appears permissible to generalize our results and to conclude that all cell nuclei, at any rate in the higher animals and probably also in the higher plants, are similarly composed. Whether or no cell nuclei contain any constant components other than those which we have termed the major ones cannot be decided from our experiments, but it is at least certain that if any do exist they must be present in such small amount as to be without significance in the formation of the main nuclear structures. As a preliminary to a consideration of the functions of the main components we now propose to discuss certain aspects of their nature and distribution.

Nucleic acid

Since the introduction of Feulgen's nucleal stain into cytological technique it has been recognized that desoxyribosenucleic acid is a constant component of cell nuclei in the higher organisms. In conformity with this view, we have isolated in the form of its sodium salt, in many cases in apparently pure form, this nucleic acid from all the cell nuclei which we have examined. Our products have been identified by their characteristic physical properties, by giving a positive Feulgen reaction and, in several cases, by analysis. This work is not, however complete and cannot, therefore, be reported on at the present time.

Basic proteins of cell nuclei

Under this term we include the proteins hitherto known as protamines and histones both of which have been observed to occur in the heads of fish sperm, while histones have also been isolated from the thymus gland of the calf and from the nuclei of avian erythrocytes (Kossel, 1928). Neither histones nor protamines have been unequivocally detected elsewhere than in cell nuclei, where they must be assumed to be in salt-like combination with nucleic acid. Both differ fundamentally in certain chemical and physiological properties, as well as in composition, from all other known proteins. The terms histone and protamine should therefore be reserved for the basic proteins of cell nuclei.

Whether the subdivision of the basic proteins of cell nuclei into two groups is justifiable or not depends upon the outlook adopted. From a physiological point of view there seems at present no reason for such a distinction for, while all cell nuclei which have been examined chemically contain a basic protein, which may be either a protamine or a histone, the two types have never been observed to occur together in the same nucleus. The occurrence of protamines appears, in fact, to be confined to the sperm heads of certain fishes. In those of others, such as the cod, a histone is found. These facts indicate that protamines and histones exercise identical functions in the nucleus so that a distinction between the two is not warranted on physiological grounds. On the other hand, from a chemical viewpoint, the distinction is a useful one, at any rate for the two protamines, salmin and clupein, which have been submitted to the most detailed investigation. These are characterised by a high content of arginine which accounts for nearly 90% of the nitrogen of the molecule, by the absence of any aromatic or sulphur-containing amino-acids and by their ability, due to their low molecular weights, to pass through dialysis membranes. In all these respects they are sharply distinguished from histones. According to our experience the latter, from whatever source they are obtained, contain much less arginine (accounting for only about 25%, sometimes appreciably less, of the total nitrogen), give a positive reaction for sulphur and free sulphydryl groups, fail to pass through collodion membranes, and give a positive test for tyrosine and a negative one for tryptophane. According to the literature (Kossel, 1928) both thymus histone and the histone from cod sperm contain tryptophane, although this author suggests that the faint reaction given by the histone from the nuclei of goose erythrocytes is due to the presence of impurity. In a recent paper Mirsky and Pollister (1946), confirming their earlier observations (Mirsky and Pollister, 1943), state that histones contain only traces of tryptophane and give the results of determinations which apparently substantiate this statement. In a footnote added during proof correction they indicate, however, that the content of tryptophane is greater than that indicated in the text. Our own statements on this subject have been based, not on actual determinations, but on qualitative tests, for which purpose we have used the sensitive mercuric sulphate-formaldehyde method. It has been our standard practice to apply this test to 10 mg. of the sulphate of the histone. When the reaction has been negative we have regarded, and still regard, the result as indicating both the purity of the preparation and the absence of tryptophane from the histone. With good preparations, including those

from the thymus gland and cod sperm, the test has consistently given completely negative results. Occasionally, however, as has sometimes happened with preparations of thymus histone, a faint positive test has been obtained. Such preparations have been judged to be impure and have been rejected as unsuitable for analytical work without further purification.

The chemical differences, enumerated above, between protamines and histones have been of great service to us in establishing (Stedman and Stedman, 1944) the nature of the basic proteins in various cell nuclei of the salmon. It has long been recognized that the sperm heads from this species contain a protamine as basic protein and that no histone is present in them. No evidence has, however, hitherto been available as to the nature of the basic protein in the remaining cell nuclei. In view of the probability, to which we have drawn attention above, that protamines and histones exercise identical functions in the cell nucleus, we at first inclined to the opinion that the chemical differences between the two types of basic proteins were merely expressions of the species difference which exists between corresponding proteins of different species. This opinion seemed to be supported by the fact that, whereas the sperm heads of the salmon and the herring contained protamine as sole basic protein, those of the cod contained a histone. We therefore expected that the basic proteins of cell nuclei of the salmon would be uniformly of the protamine type and probably identical with that in the sperm heads. It is true that Miescher, in his original work on salmon sperm, had failed to detect any protamine in material obtained from the unripe testes of the salmon so long as no spermatozoa were present. But such an experiment is difficult to repeat, for spermatozoa make their appearance at a very early stage in the development of the salmon testes. Thus, on one occasion, we were able to obtain the immature testes from a 20 pound salmon. Their degree of immaturity can be judged from the fact that they weighed only 39 g. whereas the fully developed testes of a salmon of this size would, according to Miescher's data, weigh about 500 g. Nevertheless, despite their immaturity, histological examination of the testes revealed the presence of a considerable proportion of fully developed spermatozoa among the various cells present. Examination of these testes enabled us, however, to ascertain the nature of the basic protein contained in the sperm mother cells. The mixed nuclei isolated from the minced testes weighed, after drying with alcohol and ether, 1.73 g. and, on extraction with dilute acid, yielded 18.5% of their weight of the sulphates of the basic proteins. In the manner in which these sulphates separated from solution, and in the fact that they gave a Sakaguchi reaction for arginine in the absence of alkali, it was evident from our experience with salmin sulphate itself that they contained, as was to be expected, protamine. Nevertheless, they gave, when tested with Millon's reagent, a positive reaction for tyrosine, the colour passing, as is usual with histone, into solution. The tryptophane reaction was negative, showing the absence of chromosomin. It was thus evident that the basic proteins isolated from the nuclei from the unripe testes consisted of a mixture of protamine and histone and that the latter came from the sperm mother cells. This conclusion, drawn from qualitative tests, was amply confirmed by analysis of the mixed sulphates

TABLE 3. ANALYSES OF BASIC PROTEINS FROM CELL NUCLEI OF THE SALMON

Source	N content of sulphate (%)	Arginine-N (% of total N)
Mature sperm heads	22.6	87.7
Nuclei from unripe testes	20.6	66.5
Erythrocyte nuclei	15.6	19.0
Liver nuclei	15.75	25.3

for total nitrogen and arginine and a comparison of the results with those obtained under the same conditions with a specimen of salmin isolated from the heads of salmon sperm prepared from ripe testes. These analytical results are given in Table 3, from which it can be seen that both the total nitrogen and the arginine nitrogen contents of the mixed sulphates are considerably lower than in salmin sulphate. If we accept Miescher's finding that, in the complete absence of spermatozoa, immature salmon testes contain no protamine, these results give conclusive proof that the basic protein in the nuclei of the sperm mother cells consists solely of a histone.

It was, nevertheless, desirable to obtain further confirmation of the suggestion raised by these experiments that the occurrence of protamine, which had previously only been found in the sperm heads of certain fishes, was, in fact, confined to these nuclei and was not the common basic protein of the cell nuclei of those species in which it was found in the sperm. We therefore isolated the nuclei from the liver cells and erythrocytes of the salmon. The basic proteins obtained from these nuclei possessed the entire character of histones. Like other histones, they formed a characteristic sulphate, contained tyrosine and gave a negative reaction for tryptophane. Moreover, analyses, which are included in Table 3, showed that their nitrogen and arginine contents were of the same order of magnitude as in histones from other species. With histones from mammalian nuclei, for example, we consistently find that the sulphates, under the conditions which we employ in their preparation, contain about 16% of nitrogen, 22% to 25% of this being due to arginine. There is thus no doubt that the basic proteins in the nuclei of the somatic cells of the salmon are

exclusively of the histone type and that protamine is confined in its occurrence to the spermatozoa.

An inspection of Table 3 will show that, while the nitrogen contents of the histone sulphates obtained from the nuclei of the liver cells and erythrocytes of the salmon are virtually identical, there is an appreciably greater content of arginine in the product from the liver. If this result is correct, it implies that each nucleus possesses a basic protein which is characteristic of the type of cell in which it occurs. Such a conclusion seems, at first sight, improbable. When, however, one recalls that the sperm heads of the salmon contain a protamine while the nuclei of the somatic and sperm mother cells contain, in its place, a histone, it becomes evident that there is no inherent difficulty in accepting this result. Nevertheless, while we have no reason for doubting our analyses, we prefer to wait until we can obtain material with which to repeat our work before regarding the result as finally established. Such wide differences in composition between the histones isolated from various nuclei from the ox certainly do not occur, but preliminary results suggest that differences, although much smaller in magnitude, do actually exist.

Chromosomin

When methods are available for the isolation in a pure form of a few grams of dry nuclei from any type of tissue, there is little difficulty in demonstrating the presence of chromosomin in them although the process is rather time-consuming. Our usual procedure has been first to remove the basic protein by exhaustive extraction with decinormal mineral acid, a process which removes a histone practically completely but, as previously pointed out, leaves an appreciable amount of protamine unextracted. The residue consists essentially of a mixture of nucleic acid and chromosomin. No tryptophane can, however, usually be detected at this stage in the residue by the mercuric sulphate-formaldehyde reagent because the addition of concentrated sulphuric acid, which is requisite for the completion of the test, decomposes the nucleic acid with the production of charred material, and this prevents the formation of the characteristic coloured product or destroys it as rapidly as it is formed. To demonstrate the presence of a tryptophane-containing protein it is necessary to remove the bulk of the nucleic acid by fractionating the mixture with dilute (0.2%) sodium hydroxide, a process foreshadowed by Miescher in his first investigation of salmon sperm heads and actually used by him in his isolation for the first time of nucleic acid. In principle, this method involves the repeated extraction of the mixture of nucleic acid and chromosomin with dilute alkali in the presence of salts such as sodium acetate. The nucleic acid which, under these conditions, is more soluble than the chromosomin in the dilute alkali is slowly and preferentially removed although, particularly when the proportion of nucleic acid present is high, the chromosomin also tends to dissolve. After the bulk of the nucleic acid has been removed, however, the chromosomin appears to become progressively more insoluble. When, finally, no further nucleic acid is visibly removed by the dilute alkali the chromosomin forms a solid which, after acidification with dilute acetic acid, thoroughly washing with water, and drying with alcohol and ether forms an insoluble powder, generally of a light yellow or cream colour, which is insoluble in dilute acids and alkalis. It then gives strong tryptophane, tyrosine and arginine reactions and contains sulphur. Such preparations are usually contaminated with a small amount of nucleic acid, the quantity present depending on the exhaustiveness with which the alkali extraction has been carried out. Thus, the preparation of chromosomin obtained from herring sperm (Norwegian race) contained, according to a phosphorus determination, 8.6% of nucleic acid. As judged by qualitative tests, however, there was considerably more nucleic acid in this preparation than in the one isolated from the other race of herrings, although the latter still contained sufficient to give a faint Feulgen reaction. In the earlier experiments in which the above method was employed for the isolation of chromosomin, considerable losses occurred owing to the tendency of this protein to dissolve in the alkali solution in the presence of nucleic acid. After considerable experience with the method, however, we believe that we have now so adapted the manipulative procedure that, apart from unavoidable mechanical losses, the yield is almost quantitative. Unless it is contaminated with considerable quantities of nucleic acid, chromosomin, prepared as described above, is insoluble in dilute acids and alkalis. It can, however, be dissolved in cold, normal sodium hydroxide, although dissolution in this solvent takes place slowly and requires from one to several days, according to the origin of the preparation, for completion. There is little doubt that this dissolution is accompanied by some chemical change for, while acidification with dilute acetic acid reprecipitates the bulk of the material, the process is associated with the production of a disagreeable odour, reminiscent of sulphur compounds, while the precipitate, in those cases in which we have examined it, now contains little, if any sulphur. Fractionation of such precipitates has yielded preparations which, as judged from the Feulgen test, are completely free from nucleic acid. But the losses of material which occur are so great that we have thought it advisable to concentrate our efforts on attempts to isolate chromosomin free from nucleic acid directly from the cell nuclei and without first dissolving it in normal alkali. While we have not yet succeeded in obtaining such a specimen, recent preparations have given so slight a Feulgen reaction that we are convinced that a short continuation of the extraction

procedure will suffice to achieve the desired result. It is because of the difficulty we have experienced in obtaining chromosomin completely free from nucleic acid that we have refrained from attempting to confirm and extend the provisional amino-acid determinations already reported elsewhere (Stedman and Stedman, 1947).

It will be convenient at this point to make some reference to the recent work of Mirsky and Pollister. At about the time when we first demonstrated the presence of chromosomin in cell nuclei, these authors (Mirsky and Pollister, 1943) were extracting nucleoproteins from various tissues which they claimed were composed entirely of protamine or histone nucleate. They emphasised, in fact, the absence from their preparations of any protein other than protamine and histone and, on the basis of their results put forward a plea against the rejection of the idea that histone and protamine provide the material of which the genes are composed. In a recent publication (Mirsky and Pollister, 1946), however, they now state that these nucleoproteins do, in fact contain a protein distinct from protamines and histones and they therefore describe their preparations as complexes and name them chromosins. Chromosins are distinguished from nucleohistones and nucleoprotamines by containing, in addition to the basic protein, a protein which is characterised mainly by its tryptophane content. To emphasize this, Mirsky and Pollister designate this protein by the symbol, Tr pr. It is, however, clear that the tryptophane contained in the Tr pr of Mirsky and Pollister, at any rate when it has been obtained from chromosins prepared from isolated nuclei, is due to the chromosomin which was described by us some four years ago (Stedman and Stedman, 1943; Stedman, 1944). With regard to the nature of the chromosins, it is evident from Mirsky and Pollister's results that these complexes, when prepared from isolated nuclei, consist, to a smaller or greater extent according to the manipulative procedure to which they have been subjected, of the whole nucleus. This follows from the fact that one of their preparations made from the nuclei of thymocytes had a phosphorus content of 3.69%, a value virtually identical with the values which we have obtained with the isolated nuclei of thymocytes themselves.

Functions of the Nuclear Components

The above revision of the older chemical results, according to which cell nuclei are composed of protamine or histone nucleate, necessarily reopens for discussion the question of the chemical nature of the chromosomes and of other nuclear structures such as the nuclear sap and the spindle into which it is transformed during mitosis. If the heads of fish sperm consisted, as has hitherto been believed, entirely of protamine (or histone) nucleate, the chromosomes would be bound to possess the same composition, for neither component could separately account for both the physiological functions and the chemical properties of these nuclear organs. The presence of the histone (or protamine) in the chromosomes would in these circumstances be necessary to provide the material for the genes, while the nucleic acid would be required to account for their basophilic properties. So long as the above simple nature of the heads of fish sperm was regarded as established, no other conception of the chemical nature of the chromosomes was, in fact, possible, and it is therefore not surprising that this view of their composition has been widely held. Nevertheless, it is evident that it leads to certain corollaries which can scarcely be sustained. Thus, it implies that the heads of fish sperm consist entirely of chromosomes, an implication which appears to have been accepted, at any rate in certain quarters (Barber and Callan, 1944). Yet, in those cases which have been examined, it is known that the sperm nucleus, on entering the cytoplasm of the ovum, swells until it attains the size of the egg nucleus, when it is seen to consist of chromosomes suspended in the nuclear sap. The sperm nucleus evidently contains both these structures and is thus not composed solely of chromosomes. That both structures are not usually apparent in the sperm head itself is clearly due to the compact nature of this nucleus, which causes the whole head to stain deeply with basic dyes. When it attains the size of the egg nucleus, the chromosomes remain in a concentrated state, while the material of which the nuclear sap is composed is relatively highly diluted. In this condition the chromosomes can be differentially stained with basic dyes and the presence of the nuclear sap consequently becomes evident.

Similarly, the conclusion, implied in the old view of the composition of the cell nucleus, that the basic protein of cell nuclei can provide the material of which the genes are composed is one which, to say the least, has never seemed satisfying. While histone is usually regarded as, and probably is, a fairly simple protein, its degree of complexity is not, in fact, known, and it might therefore be argued that it is sufficiently complex to fulfil this function. But in the sperm heads, for example, of the salmon and the herring there is no histone present. The basic protein in these cases consists entirely of a protamine, the simple nature of which is evident from its composition and from its ability to form a true solution. That such a substance could play the complex role of gene formation is highly improbable, and the suggestion that it could do so would scarcely have been made had it not appeared inevitable in view of the chemical composition assigned to the sperm heads in which it occurred. If, however, protamine is rejected as the material of which the genes are formed, so also must be histone, for it is evident that these two proteins play identical functions in the cell nucleus. This is clear from

the well established facts, which we have thought it worth while to confirm in the course of our work, that whereas in the sperm heads of the salmon and herring the basic protein is composed entirely of protamine, in those of the cod it consists solely of histone. Histone and protamine thus stand or fall together with regard to the claims which have been put forward to their chromosome or gene forming functions.

The above discussion has shown some of the inadequacies of the interpretation of nuclear structures in chemical terms based, as they necessarily have been based in the past, on the hitherto accepted composition of fish sperm heads. In particular, it has become clear that if nuclei were composed solely of histone (or protamine) nucleate, such a composition would be totally inadequate to account for the two main structures, the chromosomes and the nuclear sap, into which nuclei resolve themselves when they undergo division. It is doubtless for this reason that some authors, as we have pointed out in more detail elsewhere (Stedman and Stedman, 1947), have ignored the nuclear sap and have assumed, implicity or explicitly, that cell nuclei are composed wholly of chromosomes. With the discovery of chromosomin and the demonstration, made for the first time in 1943 (Stedman and Stedman, 1943), that cell nuclei in general, including fish sperm heads, are composed of three main constituents, nucleic acid, basic protein, and chromosomin, the difficulties in deducing the chemical nature of the two main nuclear structures largely disappear.

In carrying out this deductive process, we propose in the first place to consider the chemical nature of the chromosomes, since these are clearly the most important of the nuclear structures. There is probably general agreement that the essential component of the chromosomes must belong to the proteins, for no other known class of naturally occurring chemical compounds would be capable of possessing the properties or of existing in the variety necessary to account for their genetic functions. From the above discussion it is clear that, provided a suitable alternative is available, protamine and histone must be excluded from consideration on account of their simplicity. But there is a more compelling and decisive reason for such exclusion. As we have shown in our studies of the cell nuclei of the salmon, the occurrence of protamine is confined in this species to the sperm heads, in which there is no histone, while histones are found in the nuclei of the somatic cells and of the sperm mother cells. According to cytological theory, however, the nuclei of all the cells of a given species must contain chromosomes of the same structure and of essentially the same properties; they must therefore possess the same chemical composition. This they obviously could not do in the salmon if they were composed partly or wholly of protamine or histone, for these two proteins differ widely in composition. Unless, therefore, the cytological principle of the genetic continuity of the chromosomes is to be abandoned, both histone and protamine are, in the salmon, excluded from any essential participation in their formation. Since, moreover, the basic proteins must clearly exercise similar functions in the different species, this result must be generalized and applied to all species.

If this conclusion be accepted, as we think it must be accepted, it follows that chromosomin represents the essential material of the chromosomes. It is the only protein, apart from histone or protamine, present in cell nuclei in sufficient amount to serve this function. It is, in contrast to protamines and histones, of a complex nature such as would be expected of material forming the genes. It is present in all cell nuclei, so far as they have been examined, and certainly occurs in the sperm heads of the salmon as well as in the nuclei of the sperm mother cells, the liver cells and the erythrocytes of this species. It differs, so far as it has been examined, in composition in different species, and must be assumed, although there is as yet no evidence for this apart from that of a qualitative nature, to be identical in all the nuclei of a given species. It is, like the chromosomes, a relatively stable substance in the sense that, after treatment resembling fixation, it is capable of withstanding moderately drastic chemical treatment. All its general properties thus correspond with the assumption that it is, as its name is meant to indicate, the protein of the chromosomes.

The chromosomes are, however, characterised by their intensely basophilic properties, and the question therefore arises as to whether chromosomin possesses such properties. A complete answer to this question cannot at present be given, for a detailed study of its behaviour towards basic dyes has not yet been made. It is, however, certain that isolated and hence denatured chromosomin does behave towards haematoxylin (Mayer's haemalum) in the same way as do the chromosomes. When treated with this stain under the conditions employed in pathological laboratories it assumes a deep blue colour similar to that of chromosomes stained with the same dye. The dye is, moreover, firmly held, repeated washings of the stained material with water failing to remove more than traces of the dye. A specimen of chromosomin stained with haematoxylin has, in fact, been kept suspended in water for a year without a detectable quantity of colour passing into the liquid. There is thus no doubt that, as regards this stain, chromosomin corresponds in its behaviour to the chromosomes.

In the past the basophilic properties of the chromosomes have, however, been attributed exclusively to the nucleic acid which has been assumed to be present in these organs. This idea no doubt originated from the view that the heads of fish sperm were composed entirely of histone (or prota-

mine) nucleate. The basic properties of the two proteins involved are so great that they would be expected to react with acidic rather than with basic dyes. It was therefore necessary to attribute, in the main no doubt correctly in this case, the basophilic properties of sperm heads to the nucleic acid which they contained. Since the sperm heads were bound to contain chromosomes, the basophilic properties of these structures were also assumed to be due to the presence of the same material. Such a view appeared, moreover, to receive decisive confirmation when Feulgen's nucleal stain was introduced into cytological technique. In dividing cells, the chromosomes were differentially stained and the conclusion was drawn that the whole of the nucleic acid was contained in the chromosomes. In other words, it was assumed that Feulgen's technique not only detected nucleic acid in the nucleus but also located its actual position there, the inference being that any structure which contained this acid would become stained during the treatment with the reagent. We have on several occasions contested this assumption but attempts to refute our views have been repeatedly made (Callan, 1943; Barber and Callan, 1944; Serra, 1943; Brachet, 1946, 1947) although little serious attention has been given to our arguments. It is therefore necessary for us to restate our position and to discuss the relationship of Feulgen's reaction to the presence of chromosomin in the chromosomes. Feulgen's nucleal stain depends upon the presence of nucleic acid in the nucleus. On hydrolysis of sections or other preparations with hydrochloric acid this nucleic acid is partly or wholly hydrolysed. The hydrolysis is believed to remove the purine groups from the molecule, thereby unmasking aldehyde groups, although it is not excluded that a much more drastic degradation results. In any case it appears certain that aldehyde groups are, in fact, liberated and these react with Feulgen's reagent to form a violet or purple dye. Whatever the actual mechanism of the reaction may be, however, it is a certain and irrefutable fact that the dye is completely soluble in water and is therefore capable of diffusing through a solution. If any doubt remains about this statement it can be readily resolved by hydrolysing a specimen of pure nucleic acid in a test tube and adding Feulgen's reagent to the neutralised solution, a process which we have carried out on countless occasions with uniform results. If the soluble nature of the dye is accepted, it follows that nuclear structures can only become stained under Feulgen's technique provided they contain insoluble material which adsorbs or combines with or retains by some other means the soluble dye produced in the reaction. This conclusion is important for two reasons. Firstly, chromosomin, as isolated from cell nuclei, possesses in a high degree the capacity of adsorbing the soluble dye in question. If stirred into a solution of the dye prepared from nucleic acid in a test tube, it takes up the dye with great avidity and becomes stained in much the same way as do the chromosomes. Moreover, as shown by the fact that the stained product can be repeatedly washed with water, it retains the dye tenaciously. A washed specimen of the stained product has, in fact, been kept suspended in water for a year without more than a trace of the colour passing into solution. Such experiments give an obvious interpretation to the Feulgen reaction and offer a confirmation, if any further confirmation were required, of the role which we have attributed to chromosomin in the nucleus. Corresponding with this view of the mechanism of the Feulgen stain, chromosomes can be directly stained with the soluble dye (developed nucleal stain) produced from nucleic acid in the test tube, as was first shown by Choudhuri (1943) with pollen mother cells and, more recently, by Danielli (1947) using the giant salivary gland chromosomes. As Danielli points out, the chromosomes "picked up the Schiff base vigorously, even from solutions so dilute that the colour would not be observed unless particular care was taken." Danielli states that his results differ from those obtained with the normal Feulgen technique in one respect: the cytoplasm (? nuclear sap) also to some extent takes up the colour. This difference is doubtless due to the preliminary treatment with hot normal hydrochloric acid which the Feulgen technique necessarily involves. Such treatment would be expected to modify the properties of the cell by, for example, removing or destroying certain components. In the nucleus itself, it would remove the histone and destroy some, if not all, of the nucleic acid. It apparently does not, however, affect the chromosomes.

There are thus three established facts relating to the Feulgen reaction: (1) The effective dye is soluble in water; (2) chromosomin adsorbs this dye with great avidity; (3) chromosomes contained in sections or other preparations can be stained as effectively with developed nucleal stain, without preliminary hydrolysis of the preparation, as in the normal Feulgen technique. These facts clearly prove that, when the normal Feulgen technique is employed, the chromosomes retain the dye because it is strongly adsorbed by the insoluble chromosomin contained in the fixed chromosomes. It further follows that nucleic acid could exist elsewhere in the nucleus than in the chromosomes without its presence there being revealed by Feulgen staining. In other words, Feulgen staining would not locate such nucleic acid in the absence of insoluble material capable of adsorbing the soluble dye. The adsorption of the dye by chromosomin is not necessarily a specific reaction. Certain other insoluble structures would doubtless take up the dye. For example, nucleic acid itself, if coagulated by acid or other means into a compact sticky mass into which liquids can scarcely penetrate would be expected to adsorb the

dye, as in the model experiments of Brachet (1946). But if this happens during the staining of the nucleus it implies that a considerable proportion of the nucleic acid remains unhydrolysed and consequently produces no dye, in which case it is clear that the outcome of the staining can give no indication of the amount of nucleic acid present. Another example of the adsorption of the dye, which also illustrates its diffusibility, can be seen in the finding of Gates (1942) that over hydrolysis of plant cells during Feulgen staining results in the cell membrane taking up the dye. These considerations show that Feulgen's reaction is incapable of locating the position of nucleic acid in the nucleus; the staining of the chromosomes is clearly due to the adsorption of the dye by chromosomin just as the staining of the cell wall in Gates' experiments is due to its adsorption by the cellulose of this membrane. Nevertheless, while the reaction is no guide to the location of nucleic acid in the nucleus, it does not follow that structures which are actually stained contain no nucleic acid. It gives no proof, for example, that nucleic acid is absent from either the plant cell wall or the chromosomes, although it is customary to believe on other grounds that the former is devoid of nucleic acid while the latter contain this substance.

We have shown above that the presence of chromosomin in the chromosomes suffices to account for the genetic properties of the latter much more satisfactorily than has hitherto been possible. It also explains their basophilic properties towards two stains, haematoxylin and Feulgen's stain, in very common use. Whether or no it is capable alone, that is, without the cooperation of nucleic acid, of completely explaining the basophilic properties of the chromosomes is immaterial to our present purpose of assigning structural functions to the three main nuclear components. Having settled, indisputably as we believe, the position of chromosomin in the dividing nucleus, it now remains to consider the allocation of the two remaining constituents. In general, the dividing nucleus contains, in addition to the chromosomes, one other structure, namely, the nuclear sap. The solid matter which forms the basis of this must clearly be derived from either or both of the two constituents which we have not so far regarded as essential components of the chromosomes. That both of these components are, in fact, concerned with the formation of the nuclear sap follows immediately from the proof given above that the basic proteins do not enter into the structure of the chromosomes. It is evident from this that these proteins must be contained in the nuclear sap. But the properties of both histone and protamine are such that it is inconceivable that they could exist in the nucleus except in combination with nucleic acid. They have, moreover, been known since the original isolation of protamine from salmon sperm by Miescher and of histone nucleate from the thymus gland of the calf by Lilienfeld to be so contained in the cell. It follows that such nucleic acid as is combined with the basic proteins must also contribute to the formation of the nuclear sap. We thus reach the conclusion that, of the two structures present in the dividing nucleus, namely, the chromosomes and the nuclear sap, the former is composed essentially of chromosomin and the latter of histone (or protamine) nucleate. Since, moreover, the nuclear sap is transformed into the spindle during mitosis it follows that the spindle is also composed of histone nucleate. The universal presence in the cell nuclei of all species of a nucleic acid which, if not yet proven to be of identical composition and structure in each case, nevertheless possesses similar characteristic and perhaps unique physiochemical properties, finds its explanation in this conclusion. These properties are readily susceptible to variation and control by changes in the proportion of histone with which the nucleic acid is combined and in the salt and hydrogen-ion concentration of its environment. It is thus possible to visualise, to some extent, in terms of nucleic acid the changes in rigidity and of hydration which occur in the nucleus and which no doubt contribute to the movement of the chromosomes during mitosis. It also explains the well known gel-like properties of the spindle, which can be accounted for by no other known nuclear component.

The method we have used in deducing the composition of the nuclear structures possesses the advantage over direct cytological studies that the results can be considered to be applicable to the living cell. To form a structure such as the spindle, only a relatively small concentration of histone nucleate would be required. This nuclear organ must therefore contain a large amount of water, which would doubtless be removed in many fixing processes thereby causing the coagulation and shrinkage of the histone nucleate with its partial deposition on the chromosomes.

It does not follow from our arguments, nor have we ever claimed, that the chromosomes contain no nucleic acid. Although we believe, as we have indicated elsewhere (Stedman and Stedman, 1943), that the early prophase chromosomes consist of threads of chromosomin embedded in the nuclear sap, these threads would, presumably, be capable of combining with the nucleic acid of the latter particularly if it was present in excess of that required to combine with the available histone. Such threads would, no doubt, correspond with the so-called chromonemata, but it is conceivable that these merely represent the chromosomes in extended and perhaps diffuse form. As mitosis proceeds the threads contract by spiralisation and possibly by other means until the metaphase chromosomes, consisting of tight cylindrical spirals but usually having the appearance of rods, are formed. If we accept this view, the rod shaped chromosomes would neces-

sarily contain a core of nucleic acid, with or without histone, while the interstices between the coils would be filled with the same material and the whole would be embedded in histone nucleate. To what extent the nucleic acid, including that in the core, contiguous to and presumably combined with the chromosomin is to be regarded as an integral part of the chromosomes is a moot point. In our view, chromosomin represents the material of the permanent chromosomes, but is capable of combining with more or less nucleic acid according as the content of basic protein leaves this available. Such combination with nucleic acid would doubtless increase its adsorptive power towards some nuclear dyes although we doubt if there is much room for such an increase in the cases of haematoxylin and Feulgen's stain.

It may be objected to our view of the chemical nature of chromosomes that there is insufficient chromosomin in the sperm heads of certain fishes to account for the chromosomes which these nuclei contain. It must be remembered, however, that the nuclei formed during the early stages of the cleavage of the fertilised ovum of, for example, the salmon are very large in comparison with the chromosomes which they contain, but rapidly diminish in size as cleavage proceeds. It thus seems probable that the sperm heads of this species carry in concentrated form a reserve of nucleic acid for the use of the embyro in the early stages of its development. In any case, our conclusions regarding the function of nucleic acid in forming the nuclear sap and the spindle explain the large size of the nuclei in the early cleavage cells in terms of the large proportion of nucleic acid present in the sperm heads.

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Discussion

Brachet: You made the interesting suggestion that spermatozoa may contain an excess of DNA, which would serve as a reserve for chromosome multiplication. There is no doubt that the male pronucleus usually seems much poorer in DNA than the spermatozoon, and this change may very well not be accounted for by mere swelling of the sperm head; actually, the pronucleus looks very much like the spermatozoa which Pollister and Mirsky treated with concentrated NaCl to remove the thymonucleohistone. It would be interesting to

make quantitative measurements of the DNA content during the period between insemination and amphimixis. However, it has been shown by J. and D. Needham that the amount of DNA present in the nucleus of one single spermatozoon is negligible as compared to the RNA content of the egg; there is no doubt that any extra DNA introduced by the sperm into the egg would be completely insufficient to constitute an appreciable reserve of nucleic acid for further DNA synthesis during segmentation.

THE ACTION OF X-RAYS ON THYMUS NUCLEIC ACID

BABETTE TAYLOR,[1] JESSE P. GREENSTEIN, AND ALEXANDER HOLLAENDER

The study of the action of X-rays on isolated cellular components follows in logical sequence the observations of many workers as to the profound effects of X-rays on the viability of cells, the morphology of chromosomes, the pattern of inherited characters, and the activity of certain viruses and enzymes. If X-rays can cause mutations, and if mutations involve chromosomal material, of which thymonucleic acid is a prominent component, then it should be interesting to observe what effect X-rays have on the isolated nucleic acid itself. In undertaking such a problem it is necessary first, to establish a criterion of "activity," loss of which is both amenable to measurement and directly related to some characteristic property of the nucleic acid molecule in what can be considered to be its native state, and second, to have a sufficient quantity of carefully defined, homogeneous material with which to work.

Unfortunately, the physiologic role of nucleic acids in cells is as yet obscure, and there exists no biological or enzymatic test for assaying the degree by which one treatment or another may have altered the structure of nucleic acid so as to "denature" it. The best one can do is to isolate the material from cells in the mildest manner possible, designate as "native" the material thus isolated, and study the properties of both the native material and the denatured material which has been altered by some physical or chemical means. When nucleic acid is isolated from thymus gland by a modified Hammarsten-Bang (Hammarsten, 1924) procedure, as described below, its most characteristic property in solution is its high, anomalous viscosity. Since structural viscosity is a reflection of the asymmetry of a molecule, and in this case probably also an index of its polymerization, and can be easily and precisely measured, viscosity was the property of thymonucleic acid which we principally studied, before and after irradiation.

It has long been recognized that the viscosity characteristics of aqueous solutions of the sodium salt of thymus nucleic acid (TNA) vary considerably with the method of isolation employed. It was necessary for us to refine the method of preparation to obtain a large yield of highly polymerized, relatively salt free TNA, from which we could take samples which would give reproducible results over a long period of time. Vilbrandt and Tennent (1943) showed that the addition of acid or alkali degrades irreversibly the product which can be obtained through Hammarsten's procedure, which concerns no more drastic treatment than the use of salt and alcohol at pH 7 and at 0° C. By suitably modifying the Hammarsten method, we were able to obtain from one batch of thymus glands about 80 grams of pure white TNA fibers, whose only appreciable contaminant was sodium chloride. This material is usually hygroscopic; a dried sample yielded the following analytical values: N = 14.4%, P = 7.9%, ash = 12.01%. The N:P ratio of 1.82 corresponds to the theoretical value of 1.79 for a tetranucleotide. Preliminary analyses of this preparation indicated the presence of inorganic chloride. Whether this chloride is an integral part of the nucleic acid fiber or a contaminant remains for future investigation. The preparation was biuret negative and in 2% NaCl was shown to be a homogeneous, monodisperse system by ultracentrifugation.

For convenient reference, the details of isolation are presented:

Preparation of thymus nucleic acid (TNA)

Fifteen pounds of calf thymus glands were cleaned, finely chopped and homogenized in a Waring blender in cold distilled water, and suspended in a final volume of about fifteen liters of H_2O. This was left in the cold room overnight and then filtered through several layers of cheesecloth. The nucleoprotein was then precipitated out of the filtrate as the calcium salt, by the addition of about 20 cc. of 20% $CaCl_2$. The yield is markedly dependent on this step, an excess of $CaCl_2$ causing the precipitate to redissolve. The precipitate will settle slowly and in twelve hours about two-thirds of the volume of the preparation can be siphoned off. The remaining supernatant was centrifuged off in a Sharples supercentrifuge, the precipitate washed with cold H_2O containing $CaCl_2$ and redissolved in one liter of 10% NaCl. Once in solution, solid NaCl was added to saturation and the resulting solution poured into 12 liters of cold, saturated sodium chloride. This solution was allowed to stand in the cold for three days, to dissociate the nucleic acid from its protein component. The solution was then filtered with suction with the aid of Hyflo supercel, from which a clear highly viscous filtrate is obtained. TNA is precipitated from this filtrate with cold 95% alcohol.

We found the yield to be greatly increased if we squirted the TNA containing filtrate into large beakers of alcohol through 100 cc. syringes. As soon as the stream of filtrate under pressure hits the alcohol, TNA precipitates out in long fibers, occluding some air bubbles in the process, so that after a few minutes standing the TNA precipitate will contract and rise to the surface of the alcohol, while the salt streams to the bottom of the container. The fibrous precipitates were collected, pooled, and washed several times in 70% alcohol, then run up through 80%, 90%, 95%, and absolute alcohol and finally washed with ether. The product is white and

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fluffy and dries very quickly in air, in the form of long white fibers. It is stored in a dry bottle, but *not* in a desiccator, since a certain amount of water appears necessary to maintain the integrity of the fiber mass.

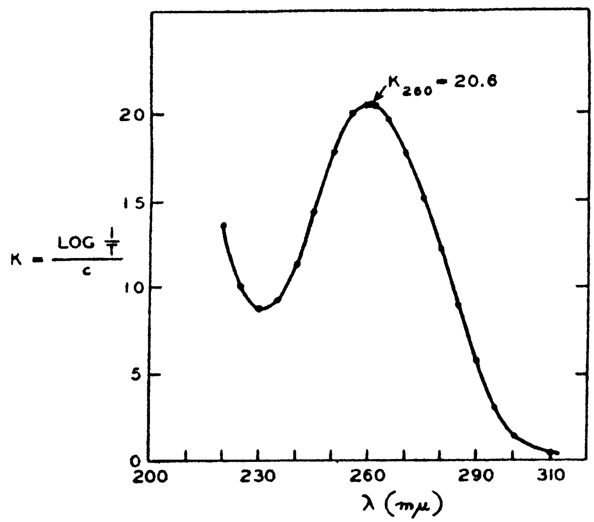

FIG. 1. Extinction coefficient curve of TNA. Concentration, .033 mgm/100 cc.

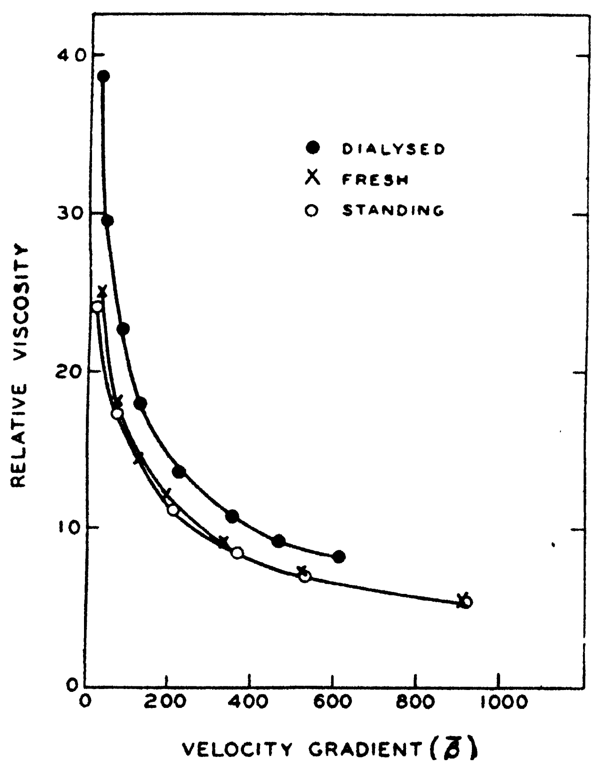

FIG. 2. Effect of standing and dialysis at 5° for 24 hours on the structural viscosity of TNA. Concentration, .2%. Dialysis against 6 liters distilled water.

When the air-dried preparation is stored in a vacuum desiccator over P_2O_5, a profound change in physical properties results. Aqueous solutions made up from such desiccator-dried preparations had a pH of about 4, as contrasted to the normal of pH 6.5, a decidedly lower viscosity, and an unusual behavior toward salt. (See below.) Consequently, this dried sample was not used for any viscosity measurements; it was only used to determine the extinction coefficient of the preparation, which constant was then utilized to calculate the concentra-

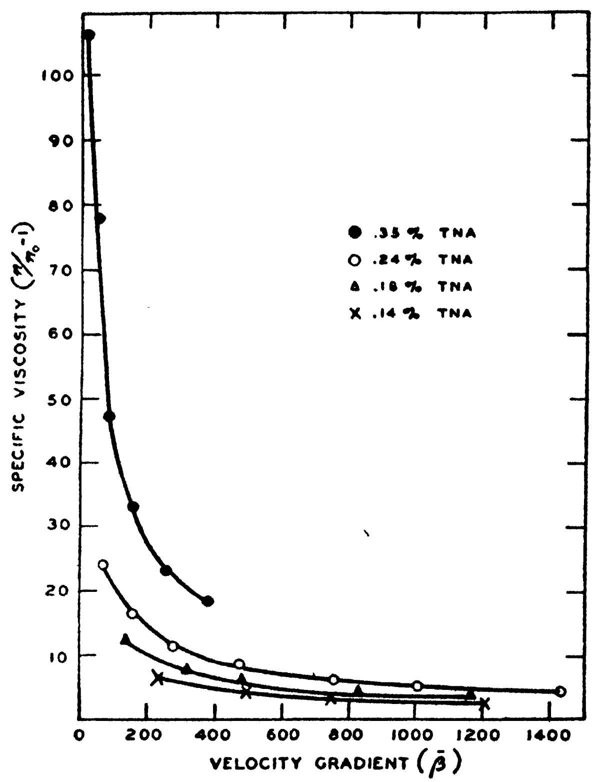

FIG. 3. Structural viscosity of four concentrations of TNA in aqueous solution.

tion of subsequent solutions of TNA made up from material which contained a variable amount of water. The curve of extinction coefficient versus wave length for a solution containing 0.033 mgm/100 cc. is given in Fig. 1, as measured on a Beckman ultraviolet spectrophotometer at maximum absorption λ = 2600 A. The value for the extinction coefficient was found to be 20.6 over a concentration range of 0.01 to 0.05 mgm/100 cc. Unknown concentrations were subsequently evaluated by diluting to the proper range, and dividing optical density at 2600 A by 20.6.

For making up solutions, the general procedure was to dissolve a weighed amount of TNA in water or salt solution; draw out the air bubbles with moderate evacuation and dialyse the solution against

6 liters of either distilled water or the required salt solution from 12 to 36 hours. This was necessary in order to insure the presence of a uniform amount of salt. The relative viscosity of solutions made up in this way was uniformly high and anomalous, *i.e.*, greatly dependent on the velocity gradient or the applied pressure. Fig. 2 shows that standing for 24 hours had no effect on the viscosity function, and dialysing only increased the absolute magnitude of the viscosity, by reducing the salt content. The family of concentration curves in Fig. 3 demonstrates how markedly the anomaly increases with small increments of concentration. We found it inconvenient to work with any concentrations above .3%, as the pressure required to force such solutions through our viscometers was too great. The viscosity of the TNA solutions was found to be were constant during the measurement to within 2 mm. H_2O. Viscosity-pressure curves were extremely anomalous, and both the height and the shape of the viscosity-pressure curves changed with changing concentrations of TNA. Consequently, for purposes of better comparison, following Edsall and Mehl (1940) we resorted to plotting viscosity against mean velocity gradient, or $\bar{\beta}$, according to Kroepelin (1929), where $\bar{\beta} = 8v/3\pi r^3 t$, since this function is independent of the measured pressure, and dependent only on the time of flow and the dimensions of the capillary involved. As shown in Fig. 4, when curves of relative viscosity against mean velocity gradient ($\bar{\beta}$) are plotted for different concentrations of TNA, the shape of the curves does not change—only the absolute magnitude of the viscosity. In

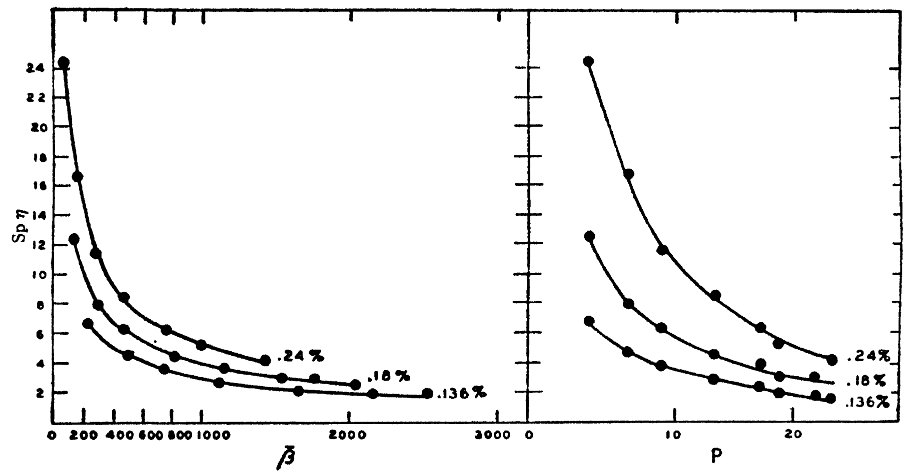

FIG. 4. Comparison of structural viscosity curves plotted as a function of pressure (cm. H_2O) and of mean velocity gradient ($\bar{\beta}$). 0.2% *TNA* in 2% NaCl solution.

insensitive to changes in pH within the range of pH 6.8-9.5. A slight drop in viscosity is noted upon acidification.

The addition of small amounts of NaCl (up to 1 M) causes a decided drop in the viscosity of control TNA solutions, has no effect on X-radiated solutions and increases the viscosity of solutions made from TNA which had been previously dried in a vacuum desiccator over P_2O_5. To these solutions the addition of 1 M NaCl is sufficient to cause gel formation. What has happened to the structure of the molecule during desiccation to be reflected in this phenomenon is not explicable at present. A certain amount of water seems to be necessary to preserve the native structure of the nucleic acid.

A brief description may be given of our method for studying viscosity. Bingham-Jackson type viscometers (Bingham and Jackson, 1918) were used, and the viscosity measured under different applied pressures, in a water bath at 30° C. The pressures used varied from 2 to 24 cm. H_2O and general, then, we considered it more valid to compare viscosities at the same velocity gradient, than at the same applied pressure (see Greenstein and Jenrette, 1940).

IMMEDIATE EFFECT OF X-RADIATION

X-radiation was delivered at 5,600 r/min. by a standard dual X-ray machine operating at a peak voltage of 180,000 volts, with 20 milliamps current through each tube. The sample was placed midway between the two tubes, whose targets were 23 cm. apart.

The anomalous viscosity noted above in solutions of TNA of concentration .2%, was completely obliterated by doses of X-radiation above 22,400 r (4 min.). As shown in Fig. 5—the relative viscosity of the X-rayed sample is not much higher than water, and completely independent of the applied pressure, or of the velocity gradient. This infers a large decrease in molecular asymmetry over the unirradiated control. The minimum amount of

X-radiation needed to completely abolish the structural viscosity was 22,400 r, as demonstrated by the curves in Fig. 6. Greater amounts of irradiation merely lowered the position of the horizontal lines.

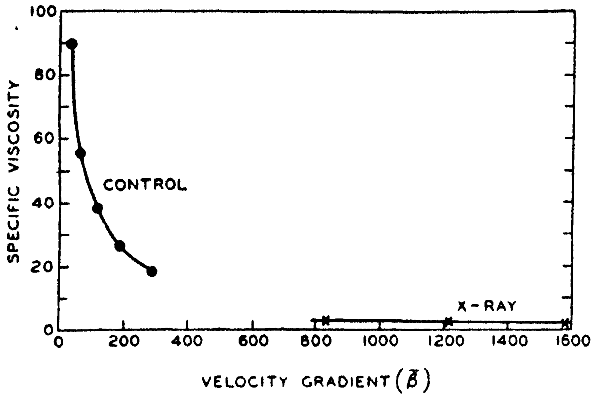

Fig. 5. Structural viscosity of 2% TNA in 2% NaCl solution before and after irradiation with 168,000 r of X-rays.

In order to obtain a dosage curve from this data, relative viscosities at constant $\bar{\beta}$ were plotted against dose of radiation, for 2 widely separated values of $\bar{\beta}$ (Fig. 7). These showed that up to 4 minutes of irradiation, the drop is linear, but after the anomaly has disappeared the drop with dosage becomes exponential, similar to the relationship which Sparrow (1946) has found.

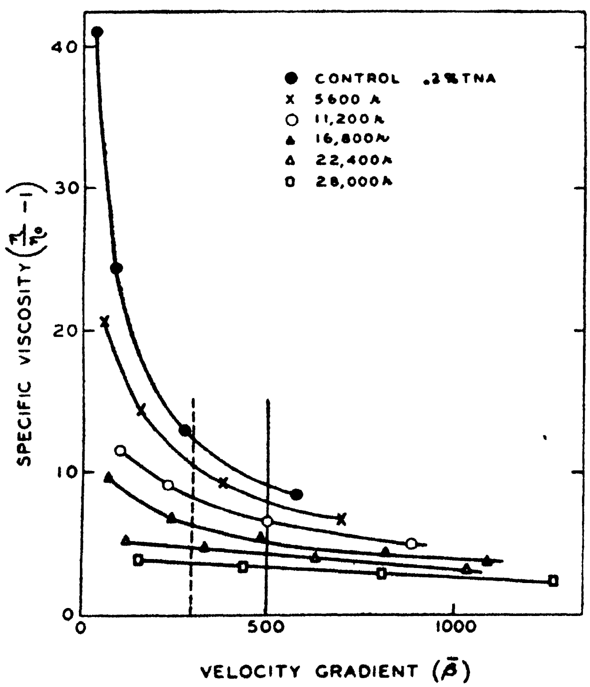

Fig. 6. Effect of various doses of X-rays on the structural viscosity of TNA. Concentration, .2% in 2% NaCl.

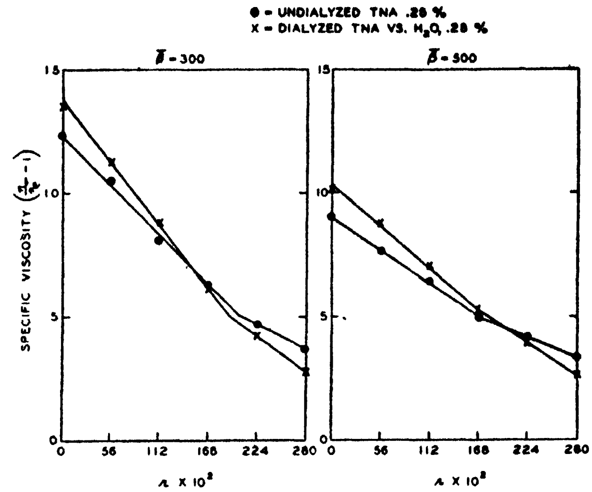

Fig. 7. Dosage curves of TNA taken at given $\bar{\beta}$'s from Fig. 6.

Spontaneous Decrease in Viscosity

Surprisingly enough, the drop in viscosity of TNA solutions initiated by X-rays continues to be expressed after cessation of the irradiation for a period of about 8 hours, when it levels off and ap-

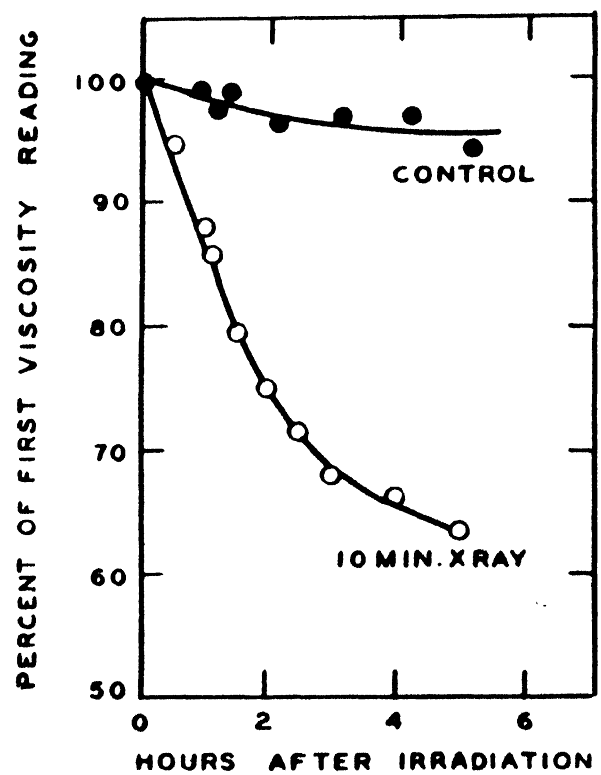

Fig. 8. Rate of decrease in viscosity of TNA in aqueous solution after the end of the irradiation period. .2% TNA, dialysed against H_2O, dosage = 56,000 r.

proaches the spontaneous drop of the control (Taylor *et al.*, 1947). When a reading of relative viscosity is taken as soon as possible after irradiation (10-30 minutes being allowed for the attainment of temperature equilibrium) and another reading is taken 4 hours later, a drop of from 20-40% of the first reading is noted. The unirradiated control solution is relatively stable, its viscosity decreasing

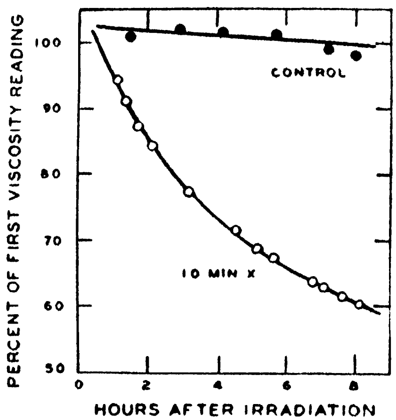

FIG. 9. Rate of decrease in viscosity of TNA in salt solution after the end of the irradiation period. .2% TNA, dialysed against 2% NaCl, dosage = 56,000 r.

from 0-5% in 4 hours. For this reason, the measurements of viscosity versus pressure, of the irradiated TNA solutions and all dosage curve measurements were performed 12 hours after the end of irradiation, after the solutions had reached a steady value.

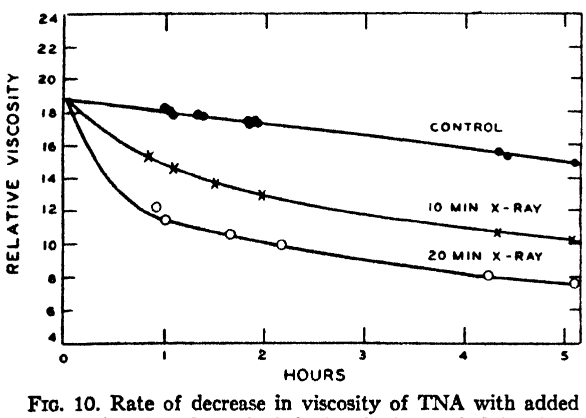

FIG. 10. Rate of decrease in viscosity of TNA with added egg albumin, after the end of the irradiation period. Nucleate concentration is .2%, protein concentration is 1%. Dosage = 56,000 r.

This subsequent fall with time after irradiation is shown in Figs. 8 and 9, in which the percentage of the first reading taken after the irradiation (usually 30 min. after) is plotted against increasing time of standing. The behavior is the same for the two preparations used, which preparations varied considerably in the method of extraction used. The presence of salt has no effect on this subsequent drop, whether the solutions were dialysed against distilled water or salt made no difference as far as the percent drop with time was concerned, although the absolute value of the control was of course

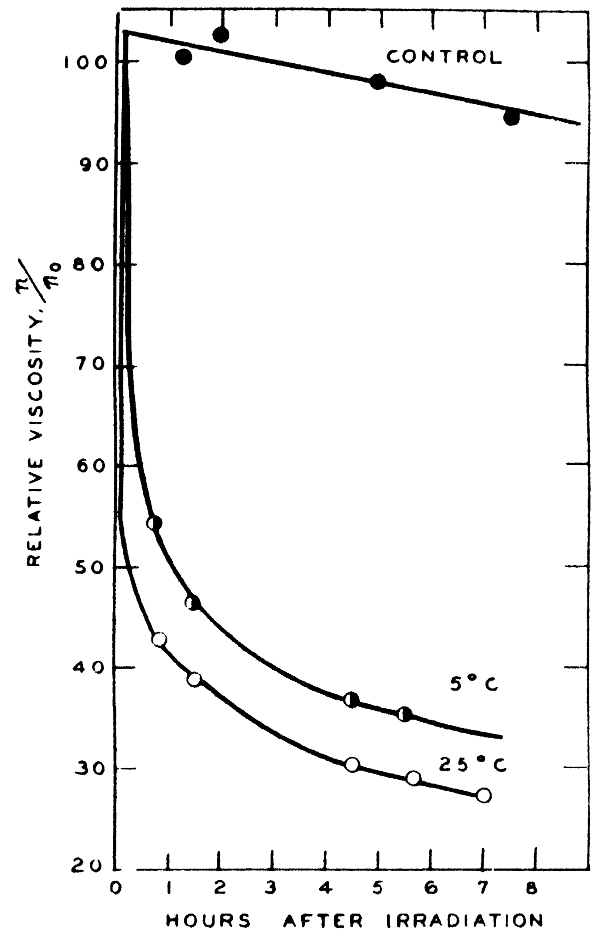

FIG. 11. Effect of temperature on the decrease of viscosity of TNA after irradiation. Dosage = 56,000 r. Concentration of nucleate = .2%. Viscosity measured at 30° C.

higher in water solution. Added protein (5% egg albumin) protected against the effect of X-rays (Fig. 10) as would be expected (see Lea, 1947), but did not affect the further drop in time. From this one can tentatively conclude that although the action of X-rays is probably both directly on the molecule itself and indirectly through "activated" water molecules, the subsequent drop in time is a reflection only of internal change in the molecule itself, concomitant to the establishment of a new equilibrium.

The Effect of Temperature

Interesting in this respect is the effect of temperature on the phenomenon. A .2% solution of TNA dialysed against water was divided into 3 aliquots: one was not irradiated, one was irradiated for 3 minutes at room temperature, and one was irradiated for 3 minutes while packed in ice, with an inside measured temperature of 5° C. The time from the end of irradiation to the beginning of the viscosity measurement at 30° was measured with a stop watch and a reading taken of the relative viscosity of each sample exactly 10 minutes after the end of the irradiation. The sample which had been irradiated at room temperature had a viscosity, relative to the control, of only 7% lower than the one which had been irradiated while cold. Immediately after the irradiation, stoppered aliquots of each of these samples were placed in the icebox at 5° C and at room temperature, for determinations of the effect of temperature on the subsequent decrease with time. Table 1 shows these data. The viscosity of both samples decreased approximately the same amount, when standing at room temperature and also when standing at 5° C. But in each case, the subsequent drop with time was suppressed by about half in the samples which stood in the cold. This would indicate that the fall in viscosity with time after irradiation was more sensitive to temperature than the direct action of X-rays on the solution of TNA. Figure 11 shows the course of the viscosity fall for a .2% TNA solution irradiated at 25° C and 5° C.

Table 1. Effect of Temperature

	Viscosity at 30° C					
	10 minutes after irradiation*		After standing 11 hours at 25° C		After standing 12 hours at 5° C	
	I		II		III	
	η/η_0	per cent drop from control	η/η_0	per cent drop from I	η/η_0	per cent drop from I
Control	13.3	—	13.4	—	13.0	—
X-radiated at 25° C	7.9	59.4%	4.8	60.8%	6.7	84.8%
X-radiated at 5° C	8.8	66.1%	5.1	58.0%	7.1	80.7%

* 16,800r in 3 minutes.

Intrinsic Viscosity of TNA

Although both the drop in the magnitude of relative viscosity and the loss of dependence on velocity gradient of solutions which have been treated with X-rays infers that the asymmetry of the molecule itself has been very much reduced by the treatment, it was decided to put the data on a basis which was independent of both velocity gradient and concentration and thus obtain a physical constant for the nucleic acid in both states which could be interpreted in terms of axial ratio. Values for the intrinsic viscosity of nucleic acid before and after irradiation were determined. Since by definition, intrinsic viscosity is $\lim_{c \to 0} \frac{(\eta/\eta_0 - 1)c}{\varphi}$ (see, for example Lauffer, 1946) where

$(\eta/\eta_0 - 1)$ = specific viscosity
c = concentration, in gms./100 cc.
φ = partial specific volume,

it is necessary to be able to extrapolate the curve of $(\eta/\eta_0 - 1)/c$ against c to 0 concentration. This can be done if the specific viscosity of the solution is proportional to the concentration. With the unirradiated nucleic acid, no such convenient proportionality was exhibited. Furthermore, in the case where the specific viscosity varies with the velocity gradient, it is necessary to extrapolate the $(\eta/\eta_0 - 1)$ against $\bar{\beta}$ curve to 0 velocity gradient in order to get a value for specific viscosity at any one concentration. In the case of nucleic acid, this curve approaches 0 asymptotically, and such extrapolation is not possible. Therefore, it became necessary to resort to a more indirect method of evaluating intrinsic viscosity. The sequence of steps in the calculation is as follows:

(1) Relative viscosity over a range of pressures from 2-24 cm. H_2O was measured on three or four dilutions of a solution of TNA, and $\eta/\eta - 1$ was plotted against c for these 4 concentrations. (see Fig. 12).
(2) At constant $\bar{\beta}$, a plot was then made of $\eta/\eta_0 - 1/c$ against concentration c. When the measurements had been done in the presence of 2% NaCl, this gave points which for each $\bar{\beta}$ fell on a straight line. Each of these lines was then extrapolated to zero concentration. (Fig. 13.)

(3) From the extrapolations in step (2) to c = 0, we then had a series of points, each representing the intrinsic viscosity of the solution *when measured at that velocity gradient.*

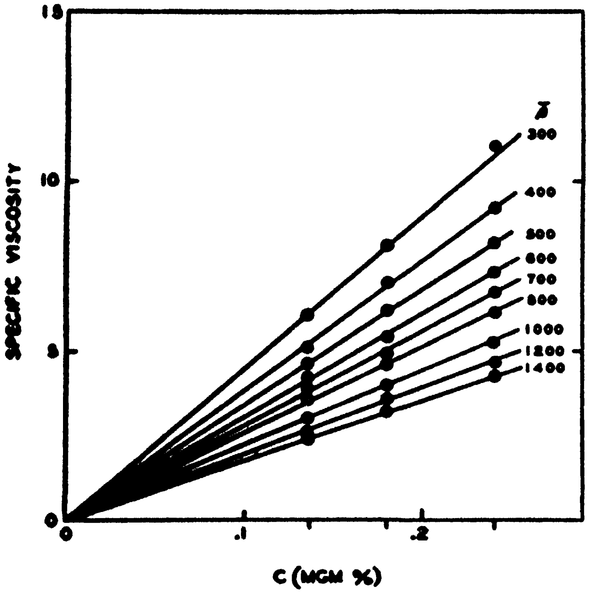

FIG. 12. Plot of specific viscosity against concentration at different velocity gradients. TNA dialysed against 2% NaCl.

These points were then plotted against concentration on a semi-logarithmic scale, and the resulting line extrapolated to zero velocity gradient. (Fig. 14.) This value divided by θ we called the

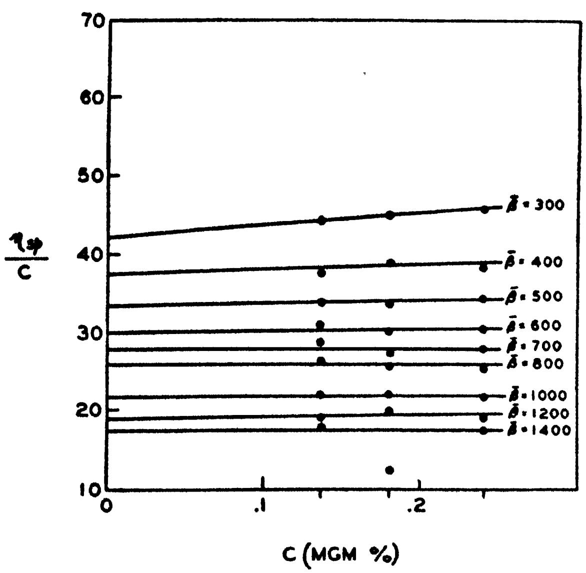

FIG. 13. Plot of specific viscosity/c against concentration at constant $\bar{\beta}$ for the concentrations given in FIG. 12, extrapolated to 0 concentration.

"apparent intrinsic viscosity" being

$$\lim_{c \to 0} \frac{\eta/\eta_0 - 1}{c} \cdot \frac{1}{\varphi} \text{ at } c = 0.$$

The value obtained in this way for TNA in water is 181 and TNA in 2% salt is 110. These values are probably much too low, on the basis of the fact that when substituted into a formula such

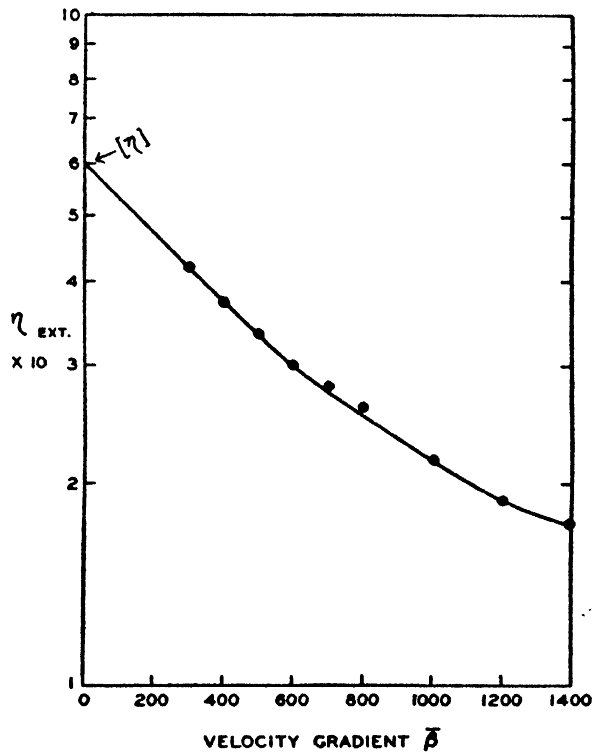

FIG. 14. Extrapolation to 0 velocity gradient of the data in FIG. 13. [η] represents intrinsic viscosity.

as Simha's (see Mehl *et al.*, 1940) for obtaining the axial ratio of the molecule, these values result in an axial ratio of 50-60, a value which, on the basis of other evidence as well as comparison with the estimates of Signer *et al.* (1938), and Tennent and Vilbrandt (1943), does not approach the right order of magnitude. It is probable that the reason for this discrepancy is the invalidity of the extrapolation to 0 velocity gradient and also the inapplicability of any equations, so far developed relating intrinsic viscosity and axial ratio, to long fibrous molecules of the type of TNA. The paucity of literature for evaluating intrinsic viscosity while taking into account the influence of velocity gradient in the measurement of relative viscosity of long asymmetric molecules is surprising. The only equation which was developed with a constant which is a function of velocity gradient is that of J. F. Lyons (1945). Unfortunately, we

have not been able to apply this equation to our data of unirradiated TNA.

On the other hand, the intrinsic viscosity of irradiated TNA could be easily evaluated. In this case, specific viscosity is roughly proportional to concentration and is independent of velocity grad-

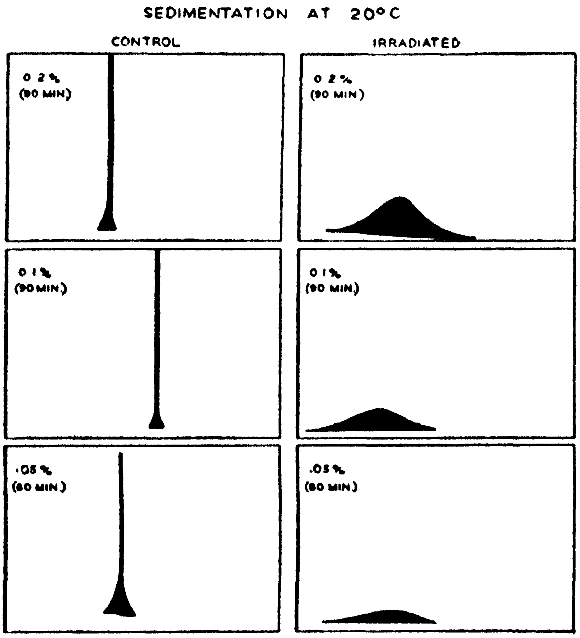

FIG. 15. Sedimentation diagrams of irradiated and control TNA, .2% in 2% NaCl, at 20° C, Tracings shown indicate sedimentation after 90 minutes.

ient. For TNA (.2%) irradiated 30 minutes, the intrinsic viscosity is close to 1.6. From Simha's equation this gives an axial ratio of about 1.

Sedimentation Data

This drop in apparent intrinsic viscosity, upon irradiation with X-rays, from 181 to 1.6 reflects a marked change in the asymmetry of the particle. But still two possibilities are open, either the original nucleic acid fiber has been broken into smaller fragments, or the original long nucleic acid fibers have associated into rounder, more symmetrical units. To answer this problem it was necessary to obtain data from an independent type of physical measurement. We are greatly indebted to Dr. Gerson Kegeles and Mr. Ed Hanson of the Department of Chemistry at the University of Wisconsin for making 6 sedimentation velocity runs for us in the ultracentrifuge. Three concentrations were run, each, of the unirradiated sample and of an aliquot which had been X-irradiated for 30 minutes (168,000 r), both in the presence of 2% salt. Pictures were taken at 30 minute intervals, with the Phillipot schlieren lens system, over a period of 2½ hours. The sedimentation was run at 20° C. In the chart in Fig. 15, are shown the tracings for each concentration taken after 1½ hours. There is a marked difference in the behavior of the two systems; the unirradiated control moves to the bottom of the cell as an essentially monodisperse system, while the irradiated sample shows a distribution which indicates the presence of many particle sizes. The sedimentation constants which Dr. Kegeles calculated were: for the control: 11 S, for the irradiated: roughly 5 S. The sedimentation for the control was a linear function of the concentration and 11 S represents an extrapolation to 0 concentration but for the X-rayed sample, no such relationship could be obtained, and 5 S represents the average constant. These data suggest that X-radiation causes a breaking of the nucleic acid particles into smaller fragments. To quote from the letter of Kegeles which accompanied the results: "It appears that the original material has a sedimentation constant in 2% NaCl at 20° C of about 11 Svedbergs. The irradiated material shows much more spreading during sedimentation and has a sedimentation constant of about 5 Svedbergs. The variation of this with concentration is irregular and is due chiefly to the error in locating the centers of such broad peaks. The irradiation appears to have affected the nucleic acid itself, reducing it to smaller particles."

Electron Micrographs

Much the same thing can be seen qualitatively from electron micrographs of the material taken before and after irradiation which Dr. Ralph Wyckoff of the National Institute of Health kindly took for us. The chromium-shadow pictures were taken of .17% TNA which had been dialysed against distilled H_2O for 24 hours. This was too concentrated a solution, and the resulting dry film had the structure of a gel. However, at the edges of the film one can see, in the case of the native

TABLE 2. COMPARISON OF PROPERTIES OF TNA BEFORE AND AFTER IRRADIATION

	Before	After
Viscosity	Anomalous; relatively stable	Newtonian; falls with time
Intrinsic viscosity (app.)	181	1.6
Flow birefringence	+++	—
Sedimentation constant	11	ca. 5
Extinction coefficient	20.6	20.6
Refractive index (.11%)	1.3363	1.3362
Dialysable-phosphate	0	0
Dialysable ammonia	0	0
Enzymatic susceptibility	high	equally high
pH	6.5	6.0
Alcohol precipitability	+	—
Acid precipitability	+	+

material, long thin fibers and in the case of the irradiated material, short, stumpy segments.

Thus the evidence gained through viscosity measurements, sedimentation, and electron microscopy all point to the fact that the effect of X-rays on solutions of sodium thymonucleate is to break the long asymmetric fibers into short fragments, of variable dimension. It was thought interesting to assay the material for chemical or enzymatic differences. These are summarized in Table 2.

Chemical and Enzymatic Studies

In order to test the possibility that inorganic phosphate or ammonia or nucleotide residues might have been split off the TNA molecule upon irradiation, 5 cc. aliquots of a .3% solution of TNA before and after irradiation were pipetted into bags of dialysing tubing which were immersed in 20 ml. of distilled H_2O in 50 cc. centrifuge tubes, and placed in an incubator at 37° (Carter and Greenstein, 1946). After a period of 4 hours, the outside dialysate was tested for ammonia, phosphate, and absorption at 2600 A. In the case of both control and irradiated material, tests for NH_3 and PO_4 were completely negative, and the amount of absorbing material which passed through the bag did not amount to more than 1% of the total material. From these experiments two conclusions were drawn: (1) that the fragmentation (depolymerization?) of TNA by X-rays does not involve the splitting of tetranucleotides to release NH_3, PO_4, or purine residues, and (2) that the molecular size on a spherical basis of the resulting fragments is over 10,000 (assuming about 20 A to be the average diameter pore of the cellophane membrane).

The absorption spectrum of the irradiated material, as measured in the Beckman spectrophotometer, corresponds exactly with that of the unirradiated control, as expected. Streaming birefringence of flow is, as Sparrow (1946) has also found, completely abolished on irradiation. We could detect no significant change in either titratable acid groups, or in acid precipitability. However, the ability to precipitate out in 95% alcohol, in the presence of salt, is completely lost in the irradiated material. Slight changes in pH were occasionally noted, the X-rayed samples sometimes dropping as much as .5 of a pH unit below the control. However, there was no regularity about this observation, and since nucleic acid exhibits practically negligible buffering power in the range of pH 6-7, these changes were considered insignificant.

Tests were made to compare the enzymatic susceptibility of the TNA solutions before and after irradiation. Samples of control and irradiated TNA were incubated with McCarty's desoxyribonuclease plus Mg^{++} according to the technique described by Carter and Greenstein (1946). There was no detectable difference in the rate of hydrolysis of the two materials. In the same way, extracts of rat spleen, high in desoxyribonuclease activity, were used to detect a difference in the substrates. Here again, both substrates were hydrolysed to the same extent and their hydrolysis was accelerated to the same extent by the addition of NaCl. These studies indicate that the chemical and enzymatic tests which are applicable to solutions of sodium thymonucleate are insensitive to the physical state of aggregation of the particle, at least within the range of particle sizes which are encountered by 20-30 minutes of X-radiation.

Conclusion

Concerning the mechanism of the action of radiations on nucleic acids, that is, whether it be direct or indirect, etc. (see Lea, 1947) we can say very little. Certainly there is a protective action exhibited by the addition of egg albumin to the solution of TNA. This, according to Lea, would indicate an indirect effect, mediated by some type of "activated" water molecules. That the effect is due to the presence of H_2O in the irradiated solvent is made improbable by the fact that the addition of H_2O, even in concentrations (10^{-2}—10^{-5}M) which far exceed those postulated for "activated" water has no effect on the viscosity of aqueous solutions of TNA.

Regardless of mechanism, the effect of X-rays on aqueous solutions of TNA is to break up the long fibrous particle into shorter fragments of variable dimensions. The passage from a state of monodispersion to a state of wide polydispersion continues, after the actual irradiation has stopped, for several hours until a new equilibrium is reached. The extent of the change, or the level of that new equilibrium depends on the dose of roentgens delivered and is independent of the time over which that dose is given. The chemical properties and enzymatic susceptibility of TNA are essentially independent of the state of polymerization or dispersion as altered by X-rays.

We wish to express our appreciation to Dr. J. M. Gonçalves for his invaluable help and advice, and also wish to thank Mr. Henry Meyer of the National Cancer Institute for his helpful cooperation with the X-ray machine.

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DISCUSSION

ZAMENHOF: Dr. Gulland has mentioned that by means of the so-called graded membranes it ought to be possible to fractionate the fragments of nucleic acids if these fragments have different molecular size.

We have prepared various graded membranes from Parlodion dissolved in acetic acid. Our aim was to separate by ultrafiltration the fragments of yeast ribonucleic acid of the size of commercial yeast ribonucleic acid, from highly polymerized yeast desoxypentose nucleic acid, in the course of purification of the latter.

With the membrane of the proper pore size it is indeed possible to obtain first drops of filtrate rich in ribonucleic acid but entirely free from highly polymerized desoxypentose nucleic acid. However, the pores very soon become clogged by the molecules of nucleic acid, with the result that the number of molecules of ribonucleic acid passing through rapidly decreases; at the same time the filtrate will be found to contain some of the highly polymerized desoxypentose nucleic acid; it should be mentioned that the passing of the latter is a matter of probability which increases as soon as its concentration increases due to the depletion of the smaller component and of the solvent.

By changing the filter after every few drops one can obtain some sort of fractionation; however, the heavy losses will probably render the method impractical.

BUSCHKE: On several occasions in today's discussions attempts have been made to compare or correlate cytological phenomena in a quantitative fashion with observations on isolated components, such as the nucleic acids, *in vitro,* and the dangers and difficulties of those attempts have been pointed out. I should like to mention some experimental observations which are perhaps relevant to this question. Ultraviolet radiation of moderate intensity causes an inhibition of mitosis in the rat's corneal epithelium which comes on with a lag period of several hours. With somewhat higher doses a more severe form of nuclear damage is produced which consists of a dissolution of the nuclear membrane and "nuclear fragmentation" eventually leading to cell death, and these changes, which can also be observed in eyes incubated after enucleation, come on with a similar lag period of about 5 hours. Now, these changes can be prevented or delayed by lowering of the temperature, and the duration of the lag period has a temperature coefficient of 3.5. Moreover, the development of these nuclear changes can be prevented by incubating the eyes anaerobically during the lag period following irradiation. This would seem to suggest that for the development of cytological effects, caused by damaging agents, the presence and functional integrity of biochemical systems, *e.g.* oxidative ones, in the cell may be required in addition to the effects observable on individual species of molecules *in vitro.*

THE RELATION OF NUCLEIC ACIDS TO THE FORMATION AND DIFFERENTIATION OF CELLULAR PROTEINS

B. THORELL

One of the fundamental processes in the development of a metazoan organism is the reproduction of its tissue cells. This involves *growth* by increase in mass and *differentiation* into functionally specialized tissue cells. The cellular growth can be defined, whether it takes place by division or by enlargement of the cell, as the formation of the fundamental substances of the cell. From a quantitative point of view, the latter consists essentially of protein substances. Thus the production of cellular proteins is the quantitatively dominating process during cell growth.

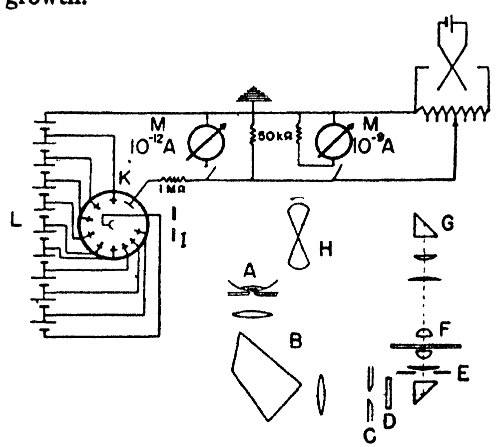


FIG. 1. Diagram of a microspectrographic measurement arrangement. A light source, B monochromator, C monochromator exit slit, D filter combination, E condenser diaphragm, F objective lens, G movable prism, H rotating sector, I photo tube diaphragm, K electron multiplier photo tube, L batteries feeding the electron multiplier tube, M high current sensitive galvanometer or mirror reflex galvanometer with lower sensitivity.

1. Survey of Methods

To attack the problems of cell physiology dealing with the relation between structure, chemical composition, and function during the growth processes, microchemical methods are necessary which permit a *quantitative* determination of the groups of substances which constitute the chief cellular constituents within parts of a cell in the living or inappreciably altered condition. In other words, methods are necessary which can measure quantities of 10^{-8} to 10^{-12} g. without greatly affecting the state of the material.

Of the procedures, differing in principle, which can be applied to cytochemical analysis, the purely optical ones seem to satisfy these needs. With them one can most simply determine substances which have a light absorption, preferably a selective one.

Perhaps more than in the case of any other quantitative analytic procedure, correct performance of the cytochemical light absorption analysis requires strictly defined conditions both as regards the material under investigation and the technical equipment. These questions will be touched on only briefly here. (For details see Caspersson, 1936, 1940a).

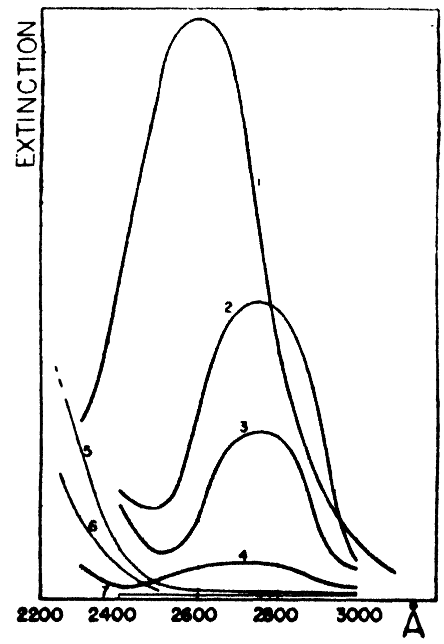


FIG. 2. Schematic representation of the main components of an ultraviolet absorption spectrum obtained from cytological material. 1. nucleic acid, 2. trytophane, 3. tyrosine (acid solution), 4. phenylalanine, 5. other amino acids, 6. Raleigh's extinction and 7. constant unspecific losses of light such as reflection and refraction. The relation between the concentrations of nucleic acid, tryptophane, tyrosine and phenylalanine is c:a 0.4:1:1:1. The other components are in arbitrary magnitudes.

It is obviously necessary, in determining the optical constants of a microscopic object with instruments which include dioptric systems, for the dis-

tribution of the intensity in the image given by the microscopic lenses to correspond to the true distribution of the intensity in the object. For a system of lenses having certain minimum apertures (>1.0) and satisfying Abbe's sine law, Caspersson (1936) showed that true absorption measurements can be made on microscopic objects whose size is not less than 3-4 times the wave length used.

These results are true only if certain conditions are controlled. The aperture is of particular importance; *i.e.*, all light passing through the object must participate in forming the microscopic image.

The errors in absorption measurements which are due to *the properties of the object* are mainly unspecific losses of light owing to reflection and refraction of boundary surfaces with discontinuities in the refraction index. These conditions must be considered for each individual case (see Caspersson, 1936, 1940a; Thorell, 1947a).

The technique of making the measurements offered several difficulties. As already mentioned, a correct absorption spectrum can be obtained only when light bundles with high aperture are used. Such lenses have high magnifications, which generally makes it necessary to work with very low light intensities in the plane of image. This condition requires first, a light source with high specific intensity of spectral pure light and, second, a very sensitive device for light-intensity measurement. I shall not enter into technical details. Fig. 1 shows the essential parts of a general microspectrographic equipment.

For the investigation of the cellular nucleic acid and protein metabolism, absorption measurement in the ultraviolet spectral range is the most important. Caspersson (1936) worked out a microspectrographic method of determination based on the high, selective light absorption of the nucleic acids in the central ultraviolet region (Fig. 2). This absorption maximum at 2600 A is due to the purine and pyrimidine bases in the nucleotides. It is practically identical for ribose- and desoxyribose nucleic acid, and is very little affected by external factors such as pH. The absorption coefficient at 2600 A is of such an order of magnitude that, with layer thicknesses corresponding to the diameter of the cells, and with the concentration of these substances in the cell, the light absorption lies within measurable range. The technique developed has rendered possible determinations of 10^{-12} g. of nucleic acid within an area of 0.2-0.5 μ in diameter.

The aromatic amino acids in the cell protein, tyrosine, tryptophane, and phenylalanine, also exhibit selective light absorption in the same wavelength range. The absorption band at 2800 A, shown by the majority of higher protein substances, is caused by these amino acids; and therefore in certain cases the amount of protein can be calculated from the light absorption data (Caspersson, 1940b).

A procedure used for localization of basic groups is founded on the observations of Chapman, Greensberg, Schmitt, 1927, who found that at very low pH certain dyes reacted with the free amino group of protein in stoichiometric relations. This fact has been used for determination of the free basic groups (Hydén, 1943b).

For the localization of nucleic acid it is also desirable to have a suitable phosphate method, and such a procedure has been worked out by Norberg (1942). With this procedure, amounts as small as 10^{-7} mg. can be determined; that is, about 10^4 times smaller than by the usual microchemical methods. During recent years a new analytical method has been developed, based on absorption microspectrography in the X-ray spectral range (Engström, 1946). This method allows the determination of a large number of elements without recourse to the combustion method. For phosphorus the new procedure is considerably more sensitive than the colorimetric procedure.

Finally, one should also mention the Feulgen reaction, which has been used to discriminate qualitatively between desoxyribose nucleic acids and ribose nucleic acids (Feulgen and Rossenbeck, 1924).

Thus, mainly by means of microspectrographic determinations of the light absorption in the central ultraviolet (3200-2400 A), it is possible to determine within individual cells the amount of polynucleotides and aromatic amino acids and to determine roughly the amount of other amino acids. It is also possible to determine roughly the distribution of basic groups, *i.e.*, the diamino acids.

2. Principal Results

These methods have rendered possible the study of the metabolism of nucleic acids and proteins within individual cells, especially during the growth processes. The results have shown on the whole that the self-reproduction system which functions in the formation of cellular proteins consists essentially of protein combined with nucleic acid.

During cell division (Caspersson, 1939; Caspersson and Schultz, 1938, 1939a) considerable changes have been observed in the nuclear desoxyribose nucleic acid concentration and distribution. During the prophase, desoxyribose nucleic acid accumulates on the chromosome elements. These observations led to the view that the reproduction of the gene elements during cell division is conditioned by the presence of desoxyribose nucleic acid.

In 1939, in the root-tips of Allium, Caspersson and Schultz (1939b) found a high concentration of ribose nucleic acid in the cytoplasm of the growth zone. On the other hand, the cells towards the base of the root had a purely tyrosine-tryptophane absorption spectrum. The facts that the formation of protein is quantitatively the main process in rapidly growing cells, and that the cytoplasm of these cells is characterized by a high concentration of

ribose nucleic acid made it appear probable that in these growing cells ribose nucleic acid is in some way linked up with cytoplasmic protein production (Caspersson and Schultz, 1939b). Later investigations, 1939-1942, on a wide range of materials—embryonic tissue, protein secreting glands, cancer tissue, growing egg cells, yeast cells, blood cells, etc.—showed the general applicability of this principle: intense protein production in the cytoplasm is always correlated with an increase in the cytoplasmic concentration of ribose nucleic acid.

The above-mentioned observations on very different materials indicated that a quite identical endocellular mechanism functions in all types of cytoplasmic protein formation (Caspersson, 1941). This can be visualized schematically in the following manner (Fig. 4):

A certain part of the chromatin, the so-called nucleolus-associated chromatin, produces substances composed of ribose nucleic acids and proteins rich in diamino acids during the telophase-interphase. These substances accumulate and form the main bulk of the nucleolus. From the nucleolus, proteins rich in basic groups diffuse towards the nuclear membrane, on the outside of which an intensive production of ribose nucleic acids takes place.

3. General Examples

I will now try to give some examples of the most essential parts of the different series of investigations, which form the basis for the above-described scheme. Analysis of *nucleolus* and *cytoplasm* in rapidly growing sea-urchin eggs showed that both these cell-organelles contain large amounts of ribose nucleic acids (Caspersson and Schultz, 1940). Similarly, in embryonic cells, nerve cells, etc., a clear parallelism was found between the nuclear mass containing ribose nucleic acid and cytoplasmic nucleic acid (Caspersson and Thorell, 1941; Hydén, 1943a; Thorell, 1944). Studies on this material showed that, apart from ribose nucleic acid, the nucleolus also contained considerable amounts of protein substances abundant in diamino acids. From these observations emerged a definition of the nucleolus based on the cytochemical properties of the nucleolar mass: a nuclear organelle in protein-generating metazoan cells which consists of ribose nucleic acid and also contains protein substances abundant in diamino acids.

The relation between the nucleolar apparatus of the nucleus and the nucleotide-protein formation system of the cytoplasm was studied in detail on ganglion cells from *Lophius piscatorius* (Fig. 3) (Hydén, 1943a). A concentration gradient of basic proteins from the nucleolus towards the part of the nuclear membrane where the formation of ribose nucleic acid in the cytoplasm takes place, indicated a migration of basic proteins from the nucleolus to the nuclear membrane.

The connection between the heterochromatin or the nucleolus-associated chromatin and the ribose nucleic acid mechanism emerged from studies on Drosophila and Chironomus material (Caspersson, 1940b, 1941; Caspersson and Schultz, 1938; Caspersson, Schultz and Aquilonius, 1940). The analysis showed that the production of nucleolar substance abundant in ribose nucleic acid and diamino acids, proceeded directly from the heterochromatic chromosome regions (Fig. 5).

In cells of higher animals also it could be shown that certain chromatin parts which differ from the main mass of the chromatin are associated with the cytoplasmic protein-forming mechanism (Caspersson and Thorell, 1941); Caspersson and Santesson, 1942; Hydén, 1943b; Thorell, 1944). In strongly growing cells these chromatin parts are characterized chemically during the interphase by the production of ribose nucleic acids, which are aggregated into a nucleolus. This chromatin was called the *nucleolus-associated chromatin* (Thorell, 1944), and in the limited number of cases where it was possible to compare this chromatin with the genetically defined heterochromatin the conceptions seem to coincide (Caspersson, 1941). The name "nucleolus-associated chromatin" was chosen to avoid the name heterochromatin, as the cytological and genetical definition of the latter in ordinary nuclei still seems to be very difficult and to some extent a matter of choice.

In the study of the growth processes in different metazoan tissue cells it is of course very rarely possible to measure, for example, the gradient from nucleolus to nuclear membrane. This is because of the small size of the cell. In the picture of a cell in intensive protein production, however, two phenomena are so evident that they cannot be missed, no matter how small the cell might be; these are the nucleolus containing ribose nucleic acids and the appearance of large amounts of ribose nucleic acid in the cytoplasm. Examples will be given below.

The most distinct protein formation occurs during embryonic growth. Fig. 6 shows embryonic liver cells. The large nucleolar mass is evident, and the absorption spectrum shows very high concentrations of ribose nucleic acid in the cytoplasm. The next figure (Fig. 7) is a section from the kidney of a chick embryo, and permits certain comparisons to be made. It has previously been shown (Fridericia, 1912) that the primitive kidney, which corresponds to an earlier phylogenetic stage, reaches maturity at the seventh day of incubation and then disappears, to be followed by the definitive kidney. Thus an individual organ reaches the adult stage while all other tissues are still in the embryonic stage. The cytochemical analysis is entirely in accordance with the view of the embryologist, that in fact this kidney passes in a few days through the whole developmental process to a true differentiated stage, and ceases to grow, at a stage when all the surrounding tissues are entirely embryonic.

4. Protein Generation in Mammalian Cells

A complete series for comparison in adult mammals of cells with different rates of cytoplasmic protein formation is given by the blood cells.

The blood cells circulating in the blood stream have a limited span of life, and new ones are continuously formed in the bone marrow. To maintain a constant number of blood cells in the blood stream a considerable rate of production is necessary; in the adult man, about 100 million erythrocytes are formed per minute. In principle the formation of new blood cells takes place from a relatively small and constant number of stem cells which produce a large number of mature blood cells by means of division and maturation through morphologically characteristic stages. The formation of mature blood cells from the stem cells of the bone marrow involves the two fundamental processes of cell renewal; partly growth by increase in size and division, and partly differentiation into a functionally specialized tissue cell.

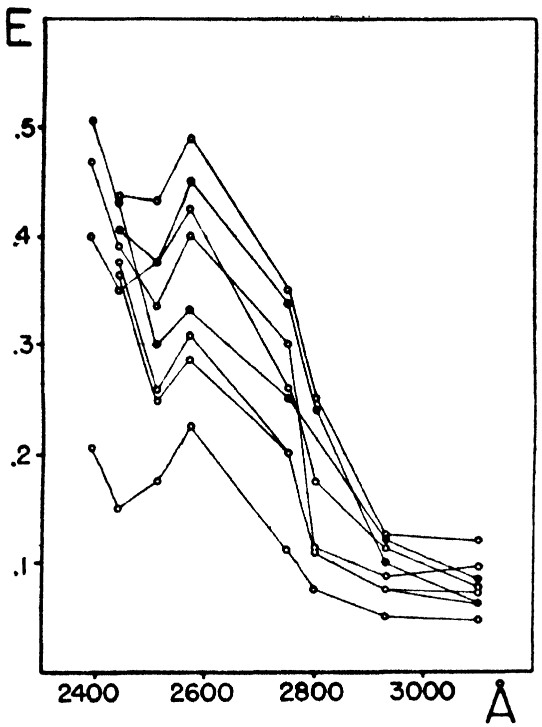


Fig. 10a

This kind of material has the great advantage that the cells are easily obtained in a living state and can thus be analyzed directly. This is especially important in the case of microspectrographic absorption analysis.

Fig. 8 shows ultraviolet microphotographs of living bone-marrow cells belonging to the granulocytic and erythropoietic series.

Fig. 9 shows the same cell types after Feulgen reaction. From these pictures it emerges that the cells in the earliest developmental stages have a Feulgen-negative nucleolus, surrounded by an outer layer of nucleolus-associated chromatin. In later, more mature stages we can find only a Feulgen-positive chromocenter in the nucleus.

Fig. 10 shows absorption spectra from points in the cytoplasm at various maturation stages in the red-blood-cell development. From these it can be calculated that the earliest stem cell has a concentration of about 5% ribose nucleic acid in its cytoplasm, which concentration very rapidly decreases

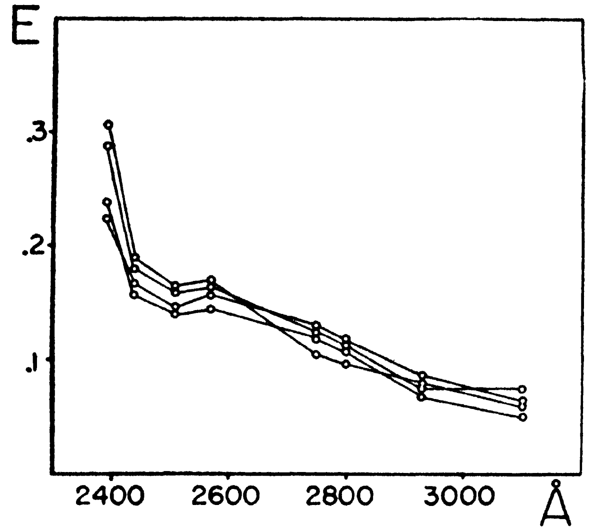


Fig. 10b

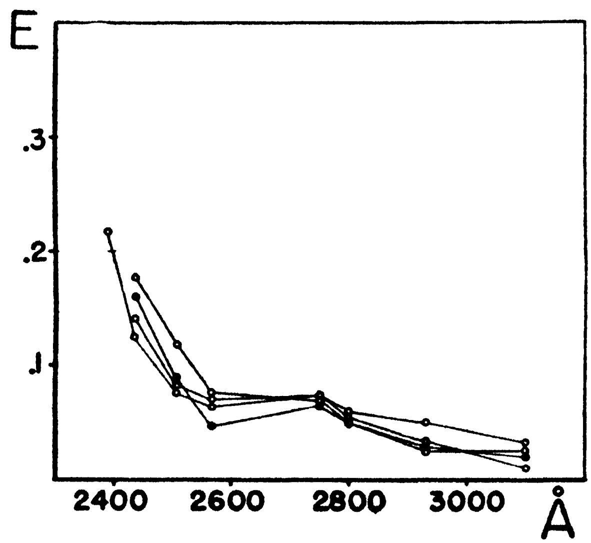


Fig. 10c

Fig. 10. Ultraviolet absorption analyses of bone marrow cells (points in the cytoplasm) at various stages of maturation. a. Stem-cell cytoplasm. b. "Basophilic" erythroblast, and c. "Orthochromatic" erythroblast.

during maturation. The cytoplasmic absorption of the final stages shows a pure tyrosine-tryptophane-spectrum.

Essentially the same results are obtained from the granulopoietic cells.

Can these cytochemical findings be correlated with the corresponding process in the generation of compared with the cytochemical changes during the red-blood-cell development. From this it appears that it is only prior to and during the principal increase in cell substance (8 times) that the cell contains a large ribose nucleic acid-containing nucleolar mass and high concentrations (>5%) of ribose polynucleotides in the cytoplasm.

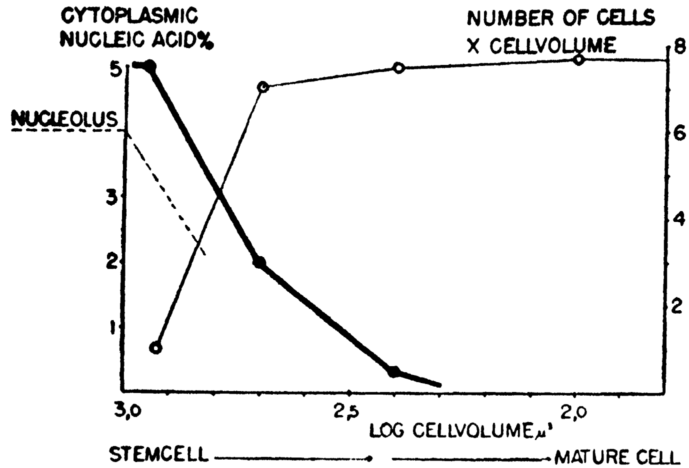


FIG. 11. Survey of some essential cytochemical and cytological changes connected with the growth processes during erythropoiesis. Abscissa: logarithm of cellular volume. (As can be calculated from cytometrical values the log cellular volume of the stem cells is c. 2, 95, basophilic erythroblast 2, 70 and orthochromatic erythroblast 2, 0). Under certain conditions the log cellular volume is proportional to time.

The figure shows that it is before and during the vastly predominating increase in mass (number of cells $\times$ cell volume), that the cells are cytochemically characterized by a large nucleolar mass and high concentrations of cytoplasmic ribose nucleic acids.

cytoplasmic proteins, *i.e.*, can the high ribose nucleic acid content of the nucleolus and cytoplasm in the earliest developmental stages be correlated with the principal increase in cell substance during blood-cell formation?

During blood-cell formation the generation of cell substance gives rise partly to a change in the number of cells, and partly to a change in cell volume. The course of this cell development can be divided into stages according to certain principles. Each phase of development is represented by a total cell volume, which is obtained by multiplying the number of cells in the phase in question by the cell volume characteristic of the phase. Thus the relation between the total volume of the different stages of development can be employed as an expression of the quantitative changes in the cell mass during blood cell development (but only if the protein concentration of the cell is constant, which within certain limits can be controlled by the ultraviolet absorption analysis). If this relation be calculated, it is found that the total volume of stem cells to that of the next phase is about 1:8. During later phases of development, the total volume is broadly speaking, constant.

In Fig. 11 these changes in cellular volume are

The effect is of such an order of magnitude that one can have no doubt in correlating the cytochemical constitution of the stem cell, characterized by high concentrations of ribose nucleic acid, with the vastly predominating generation of cell substance during the blood-cell renewal. During maturation the activity of the cell in the protein generation decreases, parallel with a continuous decrease in the concentration of ribose nucleic acid in the cytoplasm and in the nucleolar mass (for details, see Thorell, 1944, 1947a, b).

Data obtained from the white-blood-cell renewal show essentially the same relations. Some other mammalian tissues were studied and the conditions were found to be the same—for example, the continuous dentine cell formation in the incisors of the guinea pig (Thorell and Wilton, 1945).

5. The Differentiation of the Cellular Proteins Formed During Growth

Finally, a question regarding the differentiation of proteins will be dealt with. This is the relation of the specific cell differentiation to the growth of the cell, *i.e.*, the relation of the increase in cell mass to the formation of the specific cell organ, the

substances which allow the cell to perform its specific function.

The endocellular formation of the proteid-complex hemoglobin has been especially studied. As appears from what has been said before, the essential process in the growth of the tissue elements of the blood-forming organ, the generation of the fundamental protein substances of the cell-body, is correlated with the metabolism of ribose nucleic acid within certain cell organelles. From these studies it appears that the intensity of the growth processes during blood-cell maturation is proportional to the amount of nucleolar substance and the concentration of ribose nucleic acid in the cytoplasm. Conversely, we hereby possess the possibility of determining the growth conditions of the individual cell by means of analysis of the cellular concentration and distribution of the ribose nucleic acid.

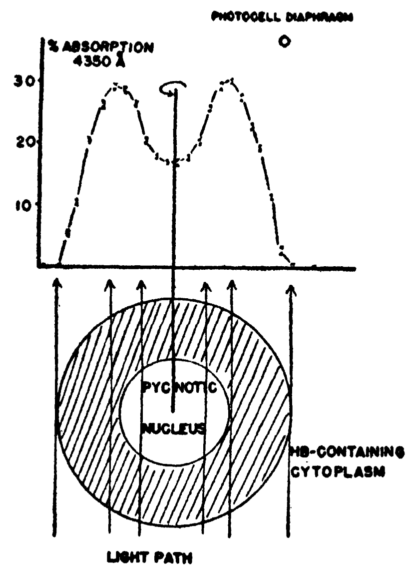


FIG. 12. The principle for hemoglobin determination in a single cell (specifically, determination of the prosthetic group). The curve shows measurements on a living "orthochromatic" erythroblast in physiological saline solution. In order to keep the hemoglobin in its oxy-form, oxygen-bubbles are present between the object slide and the cover glass. The values in the curve are obtained by moving the image of the cell with the microspectrograph prism G (fig. 1) along the cell diameter over the photo tube diaphragm I. By knowing the shape and dimensions of the cell, the hemoglobin concentration in each point along the diameter can be calculated. Unspecific losses of light must be considered. By integrating the measurement curve over the cellular volume, as indicated by the arrow, the total amount of hemoglobin in the cell can be calculated.

The protein differentiation during the formation of the red blood cells is characterized by the formation of hemoglobin. If the hemoglobin content within each individual cell is measured during the different developmental phases, a measure is obtained of the degree of differentiation of the proteins within the respective type of cells.

Thus the functional essential hemoglobin formation can be correlated with the nucleic acid metabolism of the cellular growth processes.

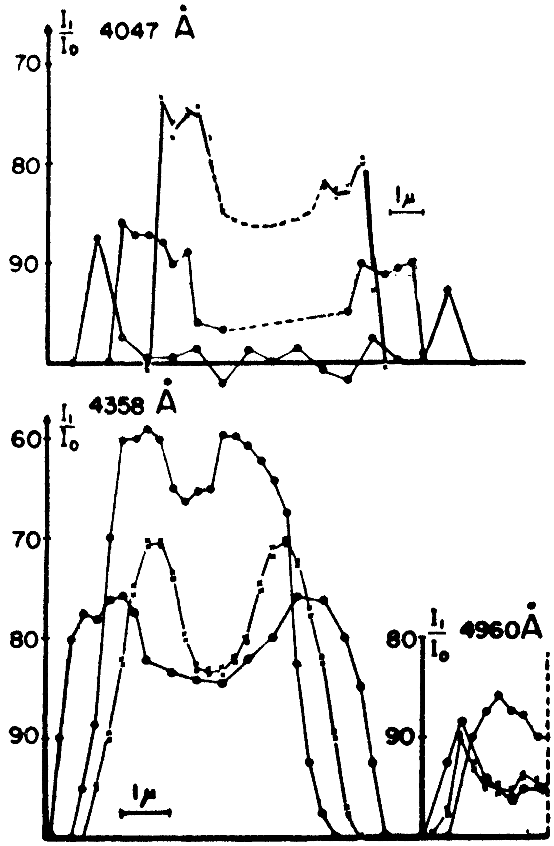


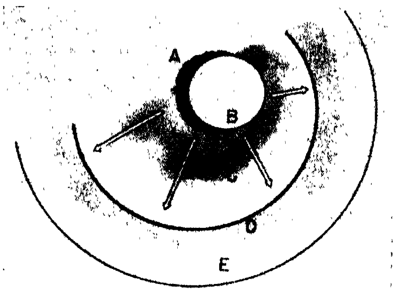
FIG. 13. Examples of absorption measurements according to the principle shown in Fig. 12 of living erythropoietic cells in physiological saline solution.

The illustration at the top represents measurements at 4047 A of early erythroblasts; proerythroblast 10.5 μ diam. —O—O—, and polychromatic erythroblasts, 8.5 μ diam. —•—•— and 7 μ diam. —X—X—. The curve obtained from the proerythroblast shows only losses of light in the periphery of the cell due to refraction. Except for this and losses of light due to a few refracting chromatin granules, the cell is optically empty. In the polychromatic erythroblasts absorbing substances appear in the cytoplasm.

The illustration at the bottom represents measurements of erythroblasts with still more hemoglobin (orthochromatic). Because of the high cytoplasmic absorption in the Soret-band, the measurements are made at 4358 A. The smaller figure shows measurement at 4960 A from which the unspecific losses of light can be evaluated.

Fig. 12 illustrates the principle for the microspectrographical hemoglobin analysis. With the methods worked out, amounts of 10^{-6} γ can be determined within individual living cells.

Fig. 13 shows light absorption analyses in the



4

3

5

6

7
DEFINITIVE KIDNEY

7
PRIMITIVE KIDNEY

FIGS. 3, 4, 5, 6 and 7.

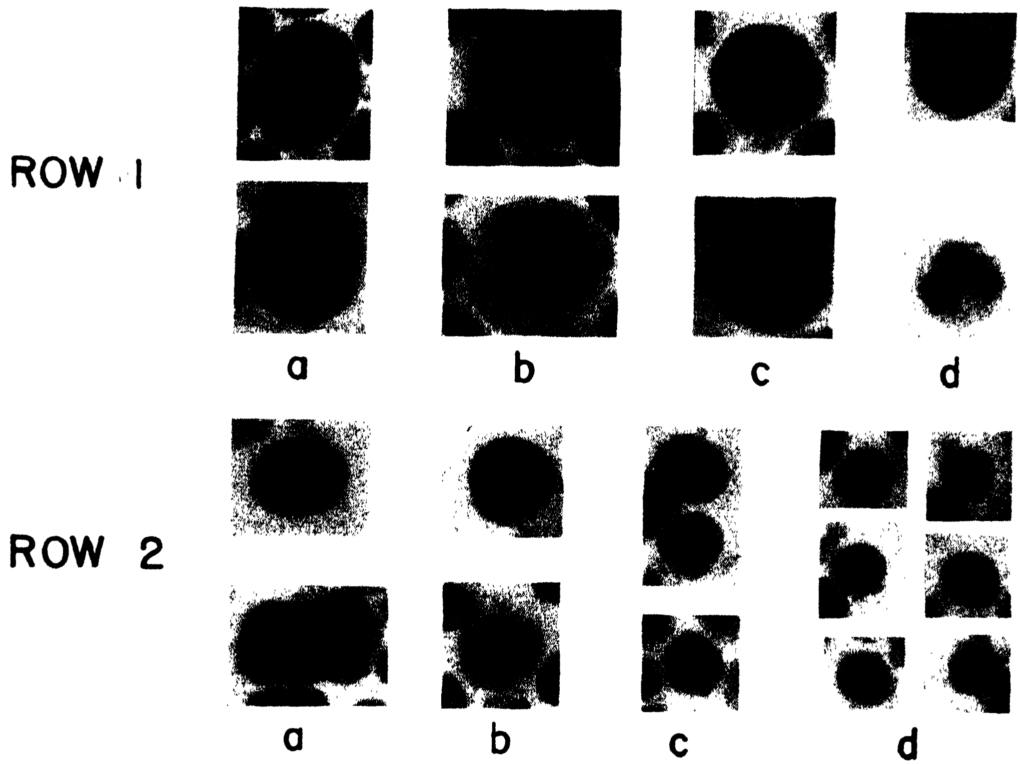
FIG. 3. Nerve cell from *Lophius piscatorius* in the stage of protein formation. Wave-length 2573 A, obj. ap. 0.85, cond. ap. 0.6 750 ×.

FIG. 4. Diagram of the endocellular mechanism for the formation of the fundamental cellular protein. A Nucleolus-associated chromatin. B. Nucleolus. C. Nuclear sap (the arrows indicate the migration of basic proteins from the nucleolar apparatus to the nuclear membrane). D. Nuclear membrane. E. Cytoplasm with ribose nucleic acid formed in the vicinity of the nuclear membrane.

FIG. 5. Fourth chromosome from Chironomus salivary gland. Ribose nucleic acid containing nucleolus formed from the heterochromatic regions. Wave-length 2573 A, obj. ap. 1.25, cond. ap. 0.9. 1000 ×.

FIG. 6. Liver cells from a 6-day chicken embryo. Frozen-dried preparation. Obj. ap. 0.85, cond. ap. 0.6. 750 ×. Wave-length 2573 A.

FIG. 7. Different parts of the kidney in the same embryo as Fig. 8.



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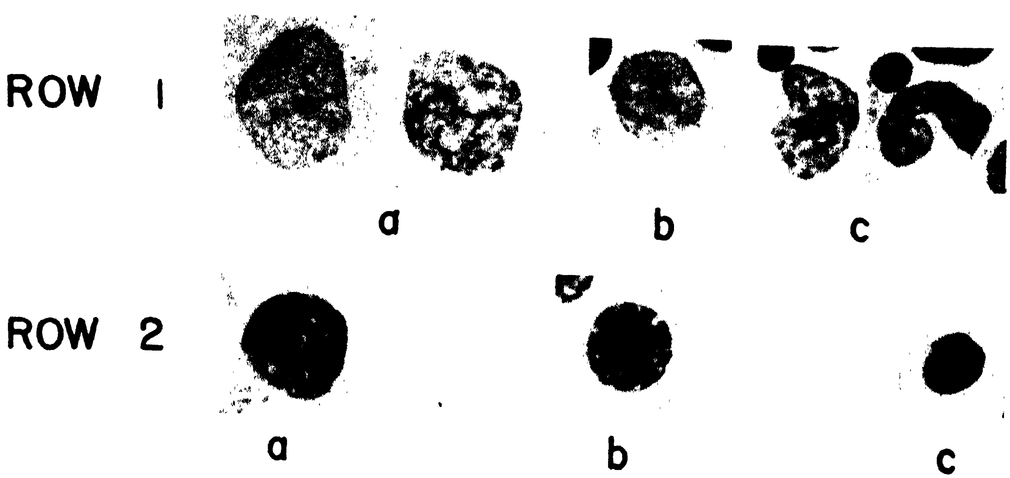

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FIG. 8. Living bone marrow cells in physiological saline solution microphotographed at 2573 A, obj. ap. 1.25, cond. ap. 0.9. Row 1: granulocytopoietic cells (1500 times enlargement). A. myeloblasts, B. promyelocytes, C. myelocytes and D. granulocytes. Row 2: erythropoietic cells (1150 times enlargement). A. proerythroblasts, B. basophilic erythroblasts, C. polychromatic erythroblasts and D. orthochromatic erythroblasts.

FIG. 9. Feulgen stained bone marrow cells. Row 1: granulocytopoietic cells A. Myeloblasts, B. promelocyte, C. myelocyte and a mature granulocyte. Row 2: erythropoietic cells A. proerythroblast, B. basophilic-polychromatic erythroblast and C. orthochromatic erythroblast. Note the disrupted nucleolus-associated chromatin in the earlier development stages (A) and the Feulgen-positive chromocentra in the mature cells.

visible spectral range, at the Soret-band of the porphyrins. By integrating the curves over the cell volume, the total amount of porphyrin substances (hemoglobin) can be calculated.

Fig. 14 shows the hemoglobin analyses together with the nucleic acid values. The quantitative changes in the cell composition during the maturation phases show, that before the beginning of the processes which lead to the final hemoglobin synthesis in the cell the metabolisms which were shown above to be connected with the increase in the cell mass have been practically completed. In the second place, it appears from the figure that the increase in the total amount of hemoglobin per cell unit does not take place uniformly. The rate of the increase is at first relatively slow, up to the phase of development which is characterized by a cell volume of $250\mu^3$. By this time the concentration of ribose nucleic acids in the cytoplasm has disappeared. Between the phases 250-$150\mu^3$ the rate of hemoglobin increase is greatest—from $1.10^{-6}\gamma$ to $23.10^{-6}\gamma$. It then stops, at a value of about $28.10^{-6}\gamma$, the same amount as an erythrocyte in the blood stream contains.

Thus on a quantitative cytochemical basis protein generation during the unipotent red-cell development occurs in several different phases, each of which is characterized by a particular cellular activity.

During the first phase, *the growth phase*, it is principally the formation of the basic cell protein substances that takes place. The cell then has a cell-chemical organization resembling that of growing cells in general—*e.g.*, embryonic cells. The next phase involves *a decline in the growth processes*, cytochemically observable as a rapid decrease in the concentration of the cytoplasmic ribose nucleic acids and the nucleolar mass. At the end of "the decline in growth," the first signs of the processes which lead up to the differentiation of the cellular proteins into their final specific forms begin to be observable. These processes become increasingly rapid, and at the phase where the concentration of cytoplasmic nucleotides reaches the zero value, an intensive production of hemoglobin sets in—*the differentiation phase*. When the cell has developed to a mature stage, the formation of hemoglobin declines—*the phase of declining differentiation*. The total amount of hemoglobin in the cell approaches a value of 28.10^{-6} γ, corresponding to that of the finished erythrocyte. Thus, in the last stage of pyknotic nuclear degeneration and during the denucleation

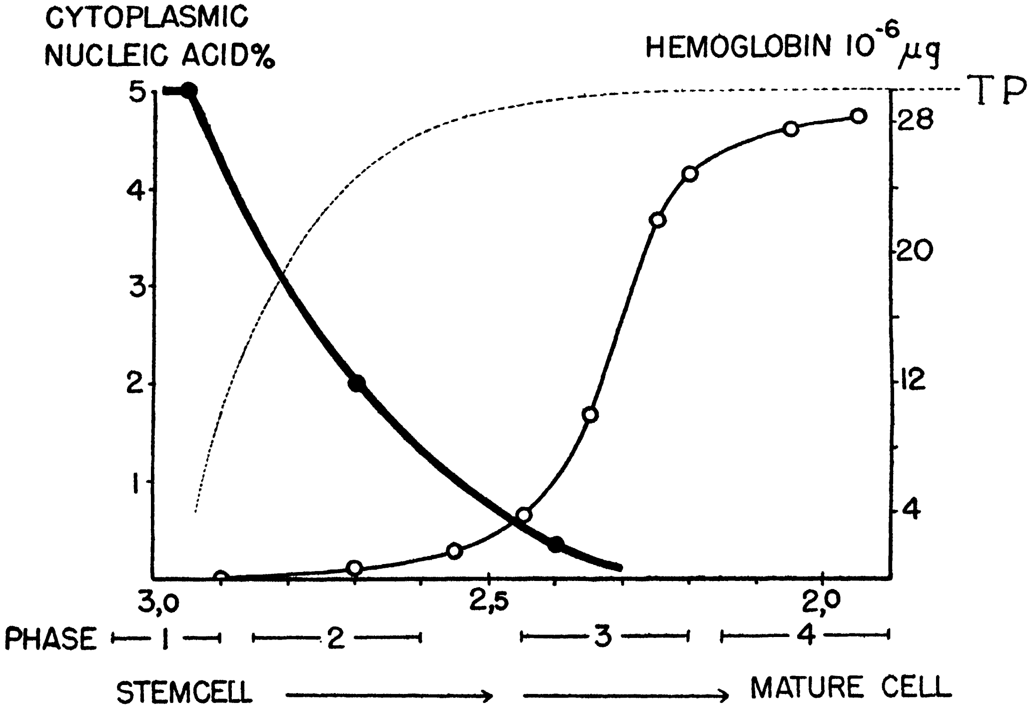

FIG. 14. Survey of the essential changes in cytoplasmic composition during erythropoiesis. Abscissa: logarithm of cellular volume (cf. fig. 11). —•—•— cytoplasmic ribose nucleic acid %, —O—O— total amount of hemoglobin in the cell, calculated from the absorption measurements in the Soret-band (cf. fig. 12 and 13). TP total cellular protein obtained from the total volume of cells in each development phase. The figure shows on the whole, that before the erythroid cell is differentiated into its final functional stage, the ribose nucleic acid metabolism associated with the endocellular growth processes is finished, *i.e.* that during the unipotent cell development the new-formation of the cellular protein substances is completed *before* differentiation occurs into its final, specific form.

process to the final erythrocyte, the hemoglobin content of the cell does not increase. The concentration becomes greater (from 25 to 33%), owing to the decrease in volume during this process.

Can this scheme of the course of the growth and differentiation processes in the generation of cytoplasmic proteins be applied in principle to other tissue cells? The regular topography of the dentine tissue in the incisors of rodents renders it possible

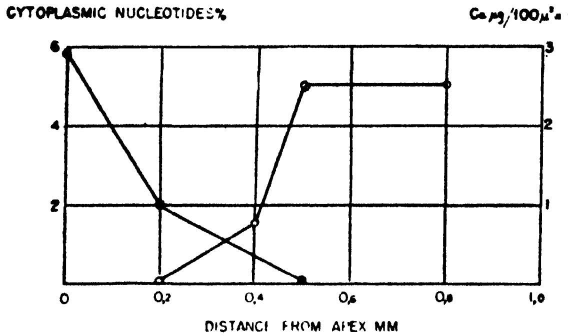

FIG. 15. The cytoplasmic composition of dentine cells in guinea-pig incisor during the development from growing root cells into mature occlusal dentine cells.

to survey cell development in the form of growth and maturation (Wilton, 1931). Microspectrographic analyses show that, during the maturation of the dentine cells a continuous decline in the function of the mechanism of the production of cytoplasmic proteins takes place (Thorell and Wilton, 1945).

The ribose polynucleotide-containing nucleolar mass and the high concentrations of cytoplasmic nucleotides, which characterize the growing cells in the root zone, disappear in the mature dentine cells.

The process which constitutes the basis of the differentiation of the dentine cells for their specific function as hard tissue, is mainly the deposition of Ca salts, conditioned by specific cytoplasmic protein substances.

The Ca content in guinea-pig incisor dentine cells during the various phases of maturation were analyzed by X-ray microspectrography (Engström, 1946). If the data from the nucleic acid analyses and Ca analyses are put together (Fig. 15), the quantitative changes which form the basis of the growth and differentiation processes during the development of the dentine cells agree well with those discussed above. The cells undergo a growth phase in the root zone characterized by an endocellular ribose nucleic acid mechanism as described earlier, followed by a phase of declining growth. During this phase the first signs of Ca deposits appear. When the cells are quite inactive from the point of view of growth—the zero value for the cytoplasmic nucleic acid—the differentiation phase begins, with a rapid increase in the Ca content. After this an equilibrium in the differentiation conditions is reached, when the Ca content remains constant.

Observations of other mammalian tissue cells under various conditions show essentially the similar results—granulocytopoesis, plasma cell development during immunization, etc. (Thorell, 1947a, b; Bing, Fagraeus and Thorell, 1945; Thorell and Wilton, 1945; Thorell, 1944, 1945; Olhagen, Thorell and Wising, 1947. This indicates that the above-described principles for the new-formation and differentiation of tissue cellular proteins during development of the unipotent stem cell are of a general character.

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DISCUSSION

STOWELL: There is one statement in the literature regarding ultraviolet microspectrophotometry which I think has been misinterpreted. Would you please clarify whether the figure of 0.1% accuracy for the photoelectric method applies to the reproducibility of readings for absorption or to the overall determinations of nucleic acids?

POLLISTER: In the usual photometric chemical analysis the non-specific light loss is cancelled out by the use of a blank which is identical with the test in every way, except that the blank lacks the specific absorbing substance for which one is testing. In the microscopic photometry which Caspersson has developed it is impossible to measure a blank; and the loss of light by nonspecific absorption must, therefore, be computed from the absorption that is found in the longer wavelengths where no specific absorption is to be expected. The accuracy of the photometric determinations is dependent upon the accuracy with which this non-specific light loss (or blank) can be computed. The estimation does not appear to be simple, since there are two sources of light loss, reflection and light scattering or dispersion. The latter is inversely proportional to the fourth power of the wavelength, and any great increase in scattering will, therefore, have a disproportionately large effect upon the determinations at the shorter wavelengths. 1) Will Dr. Thorell explain the details of how the non-specific light loss is estimated in the method? 2) Specifically will he tell us whether the curves for nucleic acid change at different stages of blood cell maturation were plotted from individual points which had been corrected for non-specific light loss? 3) Also can the ratio of light loss by reflection to that by scattering be compared for each stage of blood cell maturation, so that one can distinguish the changes in nucleic acid concentration from changes in the proportion of light scattering loss to light reflection loss? 4) Would Dr. Thorell estimate the accuracy of nucleic acid determination in this case of blood cell maturation?

THORELL: 1) The optical conditions as regards unspecific light losses by reflection, refraction and dispersion must be considered for each individual group of materials under investigation. For example, in the case of living bone-marrow cells in a physiological saline solution this can be done fairly exactly. Values of refractive index are obtained in macroscale on ground cells with an Abbe—and "dipping"—refractometer. The results are checked and the distribution of refractive index within the cells studied by an ultramicroprocedure based on the phase contrast principle. Light *reflection* in the index boundaries of the "cellular sphere" is calculated according to Fresnel's formula. In this case with index jumps of about 1.03 the light loss due to reflection will be as small as 0.7%. As the dispersion (n_F—n_C) is found to be very nearly the same for the cell substances and the medium, this value will be constant over a large wavelength range. The distribution in the microscopic image of light loss due to *refraction* is shown in the first slide in this paper. Refraction light losses can be calculated from every point in the cell with fairly good accuracy.

The light *dispersion* is most simply calculated, as Dr. Pollister mentioned, according to an approximation of Raleigh's formula. In the case of living blood cells this component of unspecific light loss is negligible except in special cases, as for example granulocytes. In these cells and in most fixed tissue sections you can fairly exactly evaluate the unspecific light losses empirically. This can be done by measuring the extinction in long wave ultraviolet and visible spectral range. You will in most cases (if no selective absorption is present) find a constant level of light loss in longer wave lengths, *i.e.,* the light loss due to reflection and refraction. In the range 3,100-3,400 the extinction increases unspecifically towards shorter wave-length: the Raleigh extinction is of a measurable order of magnitude and can be calculated from these values further down to shorter wave lengths. 2) All the curves show the primary values; they thus contain the unspecific losses of light. 3) In these measurements of living bone marrow cells I can't see any chance of such a misinterpretation. 4) In these analyses of living bone marrow cells the accuracy of nucleic acid determination is 10% to 15% of the calculated value. The accuracy of the method, *i.e.* the reproducibility of an individual measurement value, is naturally far lower, about 0.1% as mentioned by Dr. Stowell.

MICHAELIS: The question has been raised whether the corrections for the extinction curve proposed by Caspersson and his associates are legitimate. I wish to point out a criterion which shows that, for all practical purposes, those corrections are justifiable in fact. The main reason for the necessity for any corrections of the spectrophotometric readings is the fact that the scattering of light largely depends on wave length. The uncorrected readings will show a displacement of the absorption maximum of nucleic acid toward shorter wave length. On the other hand, if, after applying the corrections, the absorption maximum lies at the correct wave length as expected for nucleic acid, this very fact shows that those corrections are all right within reasonable limits of error.

MUTATIONS IN ESCHERICHIA COLI INDUCED BY CHEMICAL AGENTS[1]

EVELYN M. WITKIN

For over two decades geneticists have been interested in the possibility of inducing mutations with chemicals. Particularly, it has been hoped that mutagenic compounds might be discovered which, through their specificity of action, would lead to some understanding of the chemical basis of mutation, and ultimately of the structure and organization of the gene.

The first clearly successful attempt to induce genetic changes chemically was described by Auerbach and Robson (1944), who produced mutations and chromosomal abberrations in Drosophila by exposing the flies to mustard gas and related compounds. More recently, Demerec (1947 and unpub.) has shown that certain carcinogenic hydrocarbons (1, 2, 5, 6-dibenzanthracene, methylcholanthrene, beta naphthylamine, benzpyrene) are also effective in inducing mutations in Drosophila. These agents, like radiations, appear to be entirely nonspecific in the sense that the affected loci are distributed at random along the treated chromosomes. Nitrogen mustard has been shown to induce genetic alterations in Neurospora (Tatum, unpub.; Horowitz, Houlahan, Hungate and Wright, 1946), and in bacteria (Tatum, 1946; Bryson, unpub.), and at least one of the carcinogens, methylcholanthrene, is effective in Neurospora (Tatum, unpub.). These successful results seem to be the opening guns in what Muller called, a few years ago, "the coming *chemical* attack on the nature of the gene" (Muller, 1947). They suggest the need for a systematic survey to determine the distribution of mutagenic compounds among various chemical groups, and to lay the groundwork for subsequent analysis of their mode of action. This paper will deal with preliminary results obtained in tests of 4 substances, the first of a series to be investigated in an extensive survey.

Two methodological factors are of critical importance in an attempt to examine large numbers of compounds for mutagenic activity: the basis upon which the chemicals are selected for test, and the choice of biological material. Concerning the method of selecting chemicals, one sober if somewhat unimaginative approach is an indiscriminate raid on the nearest chemical shelf, which has the advantage of objectivity and avoidance of the hazards of premature preconceptions. On the other hand, it is far more tempting to extend oneself on the basis of present ideas concerning the possible organization of genic material, and to select chemicals which might reasonably be expected to affect, or fail to affect, the projected hereditary units. The approach used in these experiments has been to assume that nucleoproteins are somehow centrally involved in the genetic system, and, as a starting point, to investigate chemicals known to have some more or less well-defined chemical or physical effect on nucleoproteins or nucleic acids. It must be emphasized that this approach has no greater justification on *a priori* grounds than many others, and that the basis of selection may prove to be entirely spurious, since none of the chemicals tested thus far is specific in its action on nucleoproteins or nucleic acids.

The choice of biological material is obviously very important in this type of investigation. The primary requirements are 1) the availability of techniques for treating the organism with chemicals so as to be reasonably certain that they will reach and penetrate the critical sites, and 2) the availability of clear-cut genetic methods for detecting induced mutations. The penetration problem has been the most serious difficulty in the use of Drosophila for chemical induction, and although improved methods of treatment have been developed, the possibility remains that negative results may be due to the failure of some chemicals to penetrate the germ cells in sufficient concentration. The genetic techniques for detecting induced mutations in Drosophila are unparalleled in many respects, but for purposes of an extensive survey of the mutagenic action of chemicals, they are extremely laborious and slow. The problem of penetration is much less serious in microorganisms. Until recently, however, genetic methods analogous to the ClB and similar techniques in Drosophila have not been available for bacteria. At the present time, *Escherichia coli* provides promising material for a survey of the mutagenic activity of chemicals, and for detailed analysis of certain aspects of their mode of action.

Luria and Delbrück (1943) described mutants of strain B of *E. coli* which are resistant to one or more bacteriophages to which the parent strain is sensitive. These mutants arise spontaneously in cultures of the B strain at a rate of about 10^{-8} mutations per bacterium per generation, and can be detected easily by plating out samples of the culture in the presence of an excess of bacteriophage. The

[1] This work was done under an American Cancer Society fellowship recommended by the Committee on Growth of the National Research Council.

sensitive bacteria are quickly lysed, while mutants resistant to the particular bacteriophage applied appear as distinct colonies after suitable incubation.

Demerec (1946) and Demerec and Latarjet (1946) studied the effect of ultraviolet and X-rays in inducing mutations to resistance to one bacteriophage, T1, and the techniques used by them have been adapted in these experiments to the investigation of chemical mutagens. These authors showed that a certain proportion of the mutations induced by radiations are expressed immediately, before the treated bacteria undergo division. These mutations were called "zero points," as opposed to the "end-point" mutations which are also induced by the radiation, but which are expressed phenotypically only after a number of cell divisions. In the investigations to be discussed here, the effect of four chemicals on the induction of zero point mutations to resistance to bacteriophage T1 was studied.

Material

Strain B/r of *E. coli*, a radiation-resistant mutant of the B strain (Witkin, 1947), was used exclusively throughout these experiments. This strain was employed by Demerec (1946) and by Demerec and Latarjet (1946) in their studies of radiation-induced mutations, and it seemed desirable to use the same material in these investigations, in order to simplify comparison of the mutagenic action of radiation and chemicals.

The bacteriophage used to isolate resistant mutants was T1, sometimes known also as alpha, or P28. Mutants of strain B/r resistant to phage T1 are referred to as B/r/1.

Difco nutrient agar was used for all platings, and cultures were grown initially in a synthetic medium known as M-9, having the following composition per 1,000 ml. of distilled water:

KH_2PO_4	3 g.	NH_4Cl	1 g.
$MgSO_4$	0.2 g.	$Na_2HPO_4 \cdot 12H_2O$	15 g.
NaCl	0.5 g.	Dextrose	4 g.

Methods

The method used to determine the number of mutants resistant to bacteriophage T1, in both control and experimental cultures, was the standard procedure of coating the surface of agar plates with a suspension containing about 10^9 particles of T1, and then spreading 0.1 ml. of the undiluted bacterial culture on the phage-coated plate with a sterile glass rod. The plates are incubated for 48 hours at 37° C, after which time the colonies of resistant mutants are counted. The total number of bacteria per ml. of the culture is determined by plating suitable dilutions on agar, and making colony counts. The frequency of B/r/1 mutants is expressed throughout as the number per 10^8 bacteria.

The procedure for testing the mutagenic activity of chemicals has been standardized as far as possible. Variations in technique are required to allow for the peculiar properties of each chemical, and will be described in connection with the specific experiments. The basic procedure used in preliminary tests may be outlined as follows:

(1) *The preparation of suitable bacterial cultures*

Ten to 20 cultures are usually started at one time, each with a small inoculum (about 100 cells) from a stock slant of strain B/r. The cultures are grown for 48 hours at 37° C, with aeration, in a volume of 40 ml. of the synthetic medium, M-9, described above. Each culture is assayed to determine the number of bacteria per ml., and the number of B/r/1 mutants per 10^8 bacteria. The number of mutants per 10^8 bacteria in a fully grown, untreated culture is called the "background" number, and must be subtracted from the number of mutants per 10^8 survivors in the same culture after treatment with a possible mutagenic chemical, to determine the number of induced mutations. It is desirable, therefore, to use cultures having the lowest possible background number, since a high background may obscure a positive effect, particularly where the mutagenic activity is weak. Cultures found to contain more than 10 background mutants per 10^8 bacteria are discarded, and the remaining cultures are combined to form a stock pool, which is stored at 5° C, and serves to provide samples for experiments for a period of about a week.

(2) *Toxicity tests*

The optimum concentration of a chemical to be tested depends largely upon its toxicity for the bacteria. In general, for preliminary tests, a combination of concentration and time of exposure was used so as to kill about 99% of the bacteria. The high killing is an indication that the substance is penetrating the cell and reacting with its components, and 1% survival is usually just enough to permit the detection of mutants among the survivors even if no mutations are induced. The concentration or time of exposure may be adjusted later, depending upon the results of the preliminary tests. For convenience, an arbitrary time, usually 2 or 3 hours, was chosen, and the concentration of the chemical that would kill 99% of the bacteria in this period of time was determined. The following method was used.

A concentrated solution of the chemical in distilled water or a suitable buffer was prepared, and a series of widely spaced dilutions of the stock solution were made. A number of centrifuge tubes, each containing the same volume of bacterial culture, was set up and centrifuged for 20 minutes at 4,000 RPM. The clear supernatant was decanted from each tube to eliminate the nutrient medium. The bacterial pellets were then resuspended, so as to have one tube for each dilution of the chemical, and one

tube in buffer or distilled water to serve as the control. The volume of liquid in each tube was the same. The tubes were incubated for the arbitrarily chosen time, usually 2 or 3 hours, at 37° C, and then assayed to determine the number of bacteria per ml. in each of the tubes. Sometimes a second toxicity test was required, using dilutions between two of the original steps, to determine the proper concentration more precisely.

(3) *Test for mutagenic activity*

Knowing the concentration of the chemical that will kill 99% of the bacteria in 2 or 3 hours, a preliminary test is run to determine the effectiveness of the chemical under these conditions in inducing zero point mutations. Since sample experiments will be described in detail in the experimental section, only a general outline of the procedure will be given here:

A sample of a stock low-background culture is divided into two equal parts, centrifuged, and the nutrient medium decanted. One pellet is resuspended in the proper concentration of the chemical, and the other in distilled water or buffer as a control. The tubes are incubated for the length of time required to kill 99% of the bacteria in the experimental tube, and then the cultures are assayed to determine the number of viable bacteria per ml., and the number of B/r/1 mutants per 10^8 bacteria. The number of mutants per 10^8 bacteria in the control, the background number, is subtracted from the number of mutants per 10^8 survivors in the treated culture. As the background number of a given culture is extremely constant in independent determinations, seldom differing by more than 2 or 3 mutants per 10^8, an increase of 10 mutants per 10^8 over the background number is considered to be an indication of a positive effect.

Since the effect of chemicals on zero point mutations is under consideration, it is important to establish conditions under which division of bacteria during treatment will not occur. The use of a non-nutrient medium, and a concentration of bacteria at least as great as, and often 10 to 20 times greater than, the concentration reached after maximal growth, were among the precautions taken to prevent division during treatment. In addition, the exposure time was usually well below the normal lag, even for small inocula in fresh nutrient medium. Microscopic total counts revealed no measurable increase in cell number under the conditions described, over periods as long as 72 hours.

(4) *Differential survival test*

Whether the preliminary test for mutagenic activity is positive or negative, it is important to eliminate the possibility of a selective action of the chemical. If B/r/1 mutants are, for some reason, less sensitive to the toxic effects of the chemical, the proportion of mutants among the survivors of the treated culture will be higher than in the control, and a mutagenic action might be erroneously ascribed to the compound. If the mutants should be more sensitive than the nonmutants, it is possible that a positive mutagenic effect could be masked by the differential killing of mutants. Thus, to be certain that selection for or against the mutants is not responsible for an apparent positive or negative effect, it is necessary to compare the sensitivity of mutants and nonmutants to each of the chemicals tested. The standard procedure used in these experiments involved the following steps:

A stock of B/r/1 was established by inoculating a small amount of growth from about ten representative B/r/1 colonies on a control plate into a tube of M-9. A new stock was made up for each experiment, and the colonies which were isolated always came from a plating of the control tube of an experiment designed to test the mutagenic action of the chemical in question. Thus, the B/r/1 stock isolated in this way was representative of the mutants present in an experimental culture at the start of the exposure to the chemical. The culture derived from these colonies was streaked on agar to eliminate contaminating particles of bacteriophage, and another culture was started with an inoculum from at least ten colonies. This culture was used as a source of B/r/1 for the selective killing test.

To compare the sensitivity of B/r and B/r/1 to a given chemical, 48-hour aerated M-9 cultures of the two strains were grown, and equal volumes of the two cultures were mixed together, to give a culture containing approximately half mutants and half nonmutants. Two tubes were set up, each containing the same volume of the mixed culture, centrifuged, and the nutrient medium poured off. One pellet was resuspended in the standard concentration of the chemical, and the other in buffer or distilled water. The tubes were incubated for the standard time, and assayed to determine the proportion of mutants in each culture. If mutants and nonmutants are killed at the same rate, there should be no difference in the proportion of mutants between the control and experimental tubes. A sample experiment of this type will be described below.

(5) *Test of mutant colonies*

In all cases where a positive effect was obtained, samples of the mutant colonies obtained after treatment with the chemical were isolated, and tested carefully to establish the fact that they were true B/r/1 mutants. In experiments where the total number of colonies obtained was small, and where a few contaminants could distort the results, every colony was isolated and tested. The colonies were examined for resistance to T1, and also to another phage, T2, to which strain B/r is sensitive. Colonies showing resistance to T1 and sensitivity to T2 were regarded as B/r/1 mutants. Colonies showing resistance to phages were regarded as contaminants.

Experimental Results

Since the effect of chemicals in inducing zero point mutations is to be investigated, it is important to know to what extent mutations occur spontaneously, if at all, in resting cells. Luria and Delbrück (1943) have shown that mutations to resistance to T1 do not occur spontaneously in resting bacteria, and our own observations have confirmed this. Table 1 shows the results of an experiment in which resting bacteria, suspended in distilled water and incubated for a period of 24 hours at 37° C, were periodically assayed to determine the number of B/r/1 mutants per 10^8 cells. The number of mutants per 10^8 bacteria remains extremely constant under these conditions, and any mutations induced in resting cells must be compared with a baseline of zero.

Table 1. Number of B/r/1 Mutants in Resting Culture of B/r Suspended in Distilled Water for 24 Hours at 37° C.

Time after suspension in distilled H_2O	0		6 hrs.		12 hrs.		24 hrs.	
Colony Counts .05 ml. of 10^6 dilution on each of 4 plates	259		234		255		264	
	276		268		254		270	
	238		240		218		231	
	247		279		260		208	
No. bacteria per ml.	5.1×10^9		5.1×10^9		4.9×10^9		4.9×10^9	
No. B/r/1 mutants in ten 0.1 ml. samples of undiluted culture	33	41	39	23	61	37	45	50
	27	44	50	52	48	31	28	61
	28	50	31	40	32	54	37	32
	39	36	37	39	39	51	37	45
	19	40	24	41	41	45	35	36
No. B/r/1 mutants per 10^8 bacteria	7.0		7.3		8.9		8.3	

1. *Sodium Desoxycholate*

Sodium desoxycholate is a salt of the bile acid desoxycholic acid, the molecular structure of which is shown in Fig. 1, along with the structures of the other compounds tested in these experiments. It is a highly reactive compound, forming addition compounds known as choleates with a wide variety of organic substances, including fatty acids, ether, xylol and certain carcinogenic hydrocarbons (Wieland and Sorge, 1916; Fieser and Newman, 1935). Alloway (1933) used sodium desoxycholate in his early work on type transformation, and it is used by Avery and his coworkers in the preparation of transforming principle (1944), where its function is to dissolve the desoxyribose nucleoprotein complex of the pneumococcus. Mirsky and Pollister (1946) have shown that a .5% solution of sodium desoxycholate dissolves their preparations of thymus chromosin fibers, and can be used to extract the desoxyribose nucleoprotein complex from minced thymus. While this solvent action of desoxycholate on nucleoproteins served as a basis for selecting this compound for a test of its mutagenic activity, there is no doubt that it is a nonspecific effect.

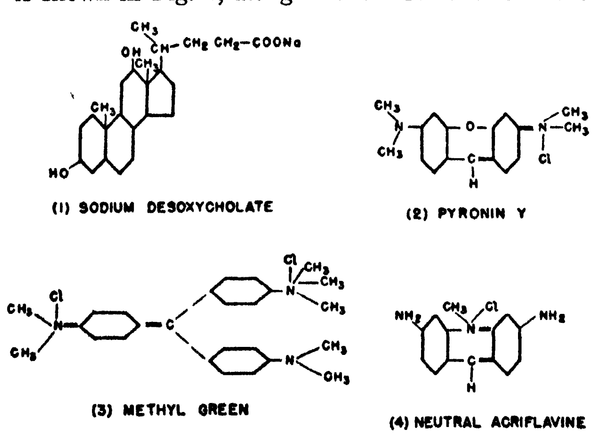

Fig. 1. Molecular structure of Sodium Desoxycholate, Pyronin; Methyl Green and Acriflavine.

Sodium desoxycholate dissolves easily in water above pH 6.5, but solutions at pH 6.5 to 7.5 form a gel at high concentrations. Since the reaction of desoxycholate in distilled water is about 7.7, unbuffered solutions of the compound in distilled water were used. Fresh solutions were made up for each experiment, and sterilized by immersion in a boiling water bath for 15 minutes.

Toxicity. Preliminary toxicity tests showed that 99% of the exposed bacteria were killed in 3 hours by a 5% solution, or in 48 hours by a 0.3% solution. All of the experiments reported here were done using the 5% solution.

Test for mutagenic activity. To illustrate in detail the method used in testing chemicals for ability to induce mutations, a typical experiment done with sodium desoxycholate will be described.

Ten-ml. aliquots of a stock low-background culture of B/r were pipetted into each of 4 centrifuge tubes, which were centrifuged for 20 minutes at 4,000 RPM. The supernatant was then decanted to

eliminate the nutrient buffered medium. The bacterial pellets in two of the tubes were resuspended in 2 ml. of a 5% solution of sodium desoxycholate, and the bacteria in the remaining two tubes were resuspended in 2 ml. of distilled water to serve as controls. In some experiments, a phosphate buffer of pH 7.7 was used for the control, but distilled water was found to be equally satisfactory. In neither case was there any change in the number of viable bacteria or in the background number during the course of any experiment. The initial centrifugation, in addition to eliminating the nutrient medium, served to concentrate the bacteria by a factor of 5 (in some experiments by as much as a factor of 20), thus facilitating the detection of mutants among the survivors.

The control and experimental tubes were incubated for 3 hours at 37° C, and were then assayed to determine the number of viable bacteria per ml., and the number of B/r/1 mutants per 10^8. The results are shown in Table 2. These results indicate the proportion of mutants among the survivors of the exposure to desoxycholate was increased from 3.8 per 10^8, the background number, to 60.2 per 10^8. Subtracting the background, the increase in mutants per 10^8 survivors is 56.4.

TABLE 2. ZERO-POINT MUTATIONS INDUCED BY 3-HOUR EXPOSURE TO 5% SODIUM DESOXYCHOLATE

1 and 2 are duplicate control tubes. 3 and 4 are duplicate experimental tubes. a and b are independent dilutions of each tube.

	Control				Experimental			
Assay								
Sample per plate	.05 ml. of 10^7 dilution				.05 ml. of 10^5 dilution			
Colony Counts	#1		#2		#3		#4	
	a. 122		a. 101		a. 79		a. 76	
	86		89		88		73	
	b. 95		b. 94		b. 101		b. 87	
	97		105		82		90	
No. bacteria per ml.	1.97×10^{10}				1.69×10^8			
No. B/r/1 mutants in samples of 0.1 ml. of undiluted culture (8 samples per tube)	#1		#2		#3		#4	
	71	59	68	83	10	11	6	8
	78	67	82	71	9	7	18	10
	67	79	74	72	14	8	4	13
	74	97	90	60	6	12	12	15
No. B/r/1 mutants per 10^8 living bacteria	3.8				Total —60.2 Induced—56.4			

Differential Survival test. In order to test the possibility that the observed increase in the proportion of mutants among the survivors of desoxycholate-treated bacteria is due to simple selection, the following experiment was done.

Cultures of B/r and B/r/1 were grown with aeration in M-9 for 48 hours, and equal volumes of the two cultures were combined in a single tube, to give a mixed culture containing approximately 50% mutants and 50% nonmutants. Ten ml. of the mixed culture was pipetted into each of two centrifuge tubes, centrifuged in the usual way, and the pellets resuspended in 2 ml. of liquid, 5% desoxycholate for the experimental tube and distilled water for the control. The tubes were incubated for three hours at 37° C. Thus, these cultures received the same treatment throughout as the cultures used to test for mutagenic activity. At the end of the 3-hour incubation, the tubes were assayed to determine the proportion of mutants in the control and experimental cultures. This was done by plating the final dilution of each culture on two series of plates, one consisting of ordinary agar plates, and one consisting of plates which had been coated previously with a heavy suspension of bacteriophage Tl. The ratio of the colony counts on phaged plates to that on unphaged plates gives the proportion of mutants in the mixture. The results are given in Table 3. In the control culture, the number of colonies developing on phaged plates was 48.9% of the number appearing on unphaged plates. In the experimental culture, in which about 99% of the bacteria were killed, the proportion of mutants among the survivors was 51.6%. Thus, it appears that mutants and nonmutants are equally sensitive to the toxic effects of sodium desoxycholate. These tests were made on mixed cultures rather than on pure cultures of the two strains in view of the possibility that competition phenomena might be involved in selective killing.

Tests of Mutant colonies. Ninety-four colonies appearing on phaged plates after treatment with desoxycholate were isolated and tested for resistance to T1 and T2. All but three proved to be

resistant to T1 and sensitive to T2, and were therefore considered to be true B/r/1 mutants. Two colonies were resistant to both phage strains, and were regarded as contaminants (in both cases the colony morphology alone rendered them suspect). The third colony was sensitive to both T1 and T2,

TABLE 3. TEST FOR DIFFERENTIAL KILLING OF STRAIN B/r AND B/r/1 BY 3-HOUR EXPOSURE TO 5% SODIUM DESOXYCHOLATE

Control = mixed culture in distilled water
Experimental = mixed culture in 5% Sodium Desoxycholate

	Control		Experimental	
Sample per plate	.05 ml. of 5×10^6 dilution		.05 ml. of 5×10^4 dilution	
Colony Counts	plated without phage	plated with excess of phage	plated without phage	plated with excess of phage
	462	192	441	233
	377	226	443	240
	468	198	482	246
	408	224	493	239
No. bacteria per ml.				
B/r+B/r/1	4.29×10^{10}		4.65×10^{8}	
B/r/1		2.1×10^{10}		2.4×10^{8}
% B/r/1	48.9		51.6	

and since this colony appeared on the edge of the plate, it is likely that it arose from a sensitive bacterium which escaped lysis due to faulty spreading of the phage suspension.

Relation between the number of induced mutations and time of exposure to 5% desoxycholate. Demerec and Latarjet (1946), in their studies of radiation-induced mutations to phage-resistance, investigated the relation between dosage of radiation and the number of mutations induced. They found that the number of zero point mutations to B/r/1 is directly proportional to X-ray dose, and bears a more complicated exponential relation to ultraviolet dose. In the present investigations, experiments were conducted to determine the analogous relation between the number of induced mutations and time of exposure to a 5% solution of sodium desoxycholate.

Fig. 2 shows a survival curve obtained with sodium desoxycholate, in which the percentage of surviving bacteria is plotted against time of exposure to the standard concentration. Fig. 3 shows the number of zero point mutations, corrected for the background number, as a function of time of exposure to 5% desoxycholate. The data upon which this curve is based were obtained by essentially the same methods as those described in the sample experiment above. Wherever possible, a single experimental tube was incubated and sampled over the entire 10-hour period, since the use of

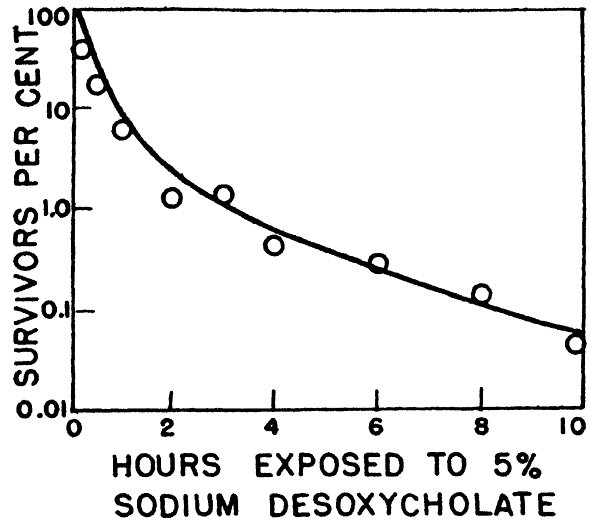

FIG. 2. Survival curve of resting bacteria exposed to 5% Sodium Desoxycholate.

separate tubes for each determination gave somewhat more variable results. The linear relation between induced mutations and exposure time can be compared directly with the X-ray dosage-effect

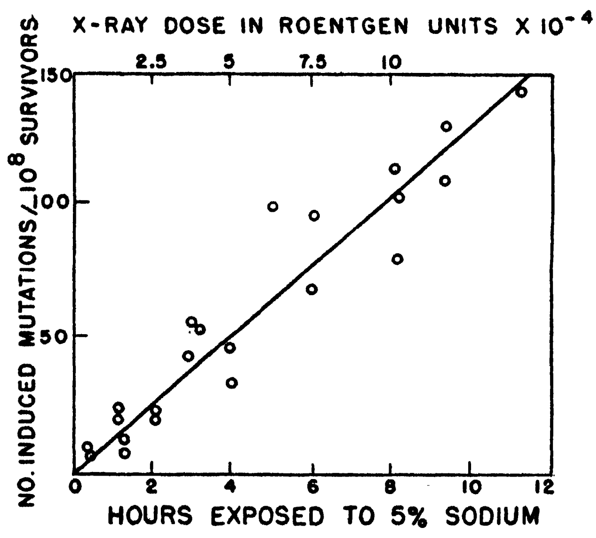

FIG. 3. Relation between the number of zero point mutations induced and time of exposure to 5% sodium desoxycholate. The upper abscissa represents X-ray dose in roentgen units, on a scale required to make this curve coincide with the curve obtained by Demerec and Latarjet (1946) for the relation between X-ray dose and number of zero point mutations.

curve obtained by Demerec and Latarjet, and differs fundamentally from their analogous ultraviolet curve. The upper abscissa in Fig. 3 represents X-ray dose in r units, on a scale required to make the desoxycholate curve coincide with the X-ray curve of Demerec and Latarjet. It will be noted that 100,000 r units correspond, in terms of the number of induced zero point mutations, to an 8-hour exposure to 5% sodium desoxycholate.

On the basis of these positive results obtained with desoxycholate as a mutagenic agent in bacteria, Demerec (unpub.) has tested the ability of this compound to induce lethal mutations in Drosophila. The proportion of lethals among the tested sperm was about 1.5%, as compared with 0.24% in controls, an effect of the same order of magnitude as that obtained with nitrogen mustard by the same technique. These results with Drosophila suggest that sodium desoxycholate may prove to be a nonspecific mutagen, like radiations and mustard. No tests have been made to investigate the possibility of specific effects on the particular mutation used as an index of mutagenic activity, namely, resistance to bacteriophage T1. For this purpose it will be necessary to investigate the action of desoxycholate on other bacterial mutations.

2. *Pyronin and Methyl Green*

Basic dyes, which stain chromatin by virtue of their affinity for nucleic acids, were considered as a class of compounds worthy of investigation for possible mutagenic activity. Pyronin and methyl green, the components of the Unna-Pappenheim mixture currently of interest in cytochemical research, were the first dyes to be tested. Brachet (1940) has described the usefulness of this mixture as a means of differentiating cytochemically between ribose and desoxyribose nucleic acids, by virtue of the selective affinity of pyronin for the ribose type of nucleic acid. Although there is good evidence for the specificity of the pyronin-methyl green mixture under certain conditions, it must be pointed out that the treatment of living bacteria, without recourse to the procedures of fixation and differentiation which are standard in cytological work, may very well present entirely different conditions. Thus pyronin may be taken up by fatty acids, or other basophilic elements ordinarily removed in cytological preparations. There is no basis, therefore, for assuming that these dyes are specific, under the conditions of these experiments, in acting exclusively upon nucleic acids, or in differentiating between the two types of nucleic acid.

Pyronin was tested by essentially the same techniques described in connection with desoxycholate. One difficulty encountered, however, was the great variability of the toxic effects of a given concentration in different experiments, or in different tubes in the same experiment. One possible source of the variability was thought to be the precipitate which forms in the presence of bacteria at high concentrations of the dye. Low concentrations, in which there is no precipitate, were tried, and in some experiments bacteria were spread on the surface of agar containing pyronin, were washed from the surface, and a concentrate of the wash was assayed. The variability of the results was not overcome by these modifications in technique, and the basis of the difficulty is not yet understood. The results obtained with pyronin, at various concentrations, and under various conditions of treatment, are summarized in Table 4. All results obtained were consistent in indicating a higher proportion of mutants among the survivors of treated bacteria than among controls. Tests for selective killing of mutants showed no difference in the sensitivity of B/r and B/r/1 to pyronin. Although these results are to be regarded as preliminary and tentative, they suggest that pyronin is active as a mutagenic agent. The problem of variability will have to be solved before any detailed quantitative analysis can be made.

Pyronin has not yet been tested on Drosophila, and there is no evidence as to its specificity in inducing mutations.

Toxicity tests with methyl green showed that

TABLE 4. ZERO POINT MUTATIONS INDUCED BY PYRONIN Y

Conc. of Pyronin Y (%)	Time of Exposure	Method of Treatment	Survival (%)	No. Induced Mutations/10^8 Survivors
0.5	20 min.	in agar	43.0	10.6
0.01	5 hr.	in liquid	32.2	22.4
0.075	1 hr.	in liquid	18.6	14.0
0.75	1 hr.	in agar	18.1	22.8
0.5	2½ hr.	in agar	12.5	33.7
0.05	3 hr.	in liquid	3.9	36.0
2.0	30 min.	in agar	2.5	41.3
0.5	15 min.	in agar	0.16	62.7
0.75	2 hr.	in agar	0.11	150.0
0.5	2 hr.	in agar	0.03	100.7
0.025	4 hr.	in liquid	0.01	1080

TABLE 5. NUMBER OF B/r/1 MUTANTS IN CULTURES EXPOSED TO 1% METHYL GREEN

Culture	Time of Exposure to 1%	Experiment #1 Survival (%)	Experiment #1 No. Mut. per 10^8	Experiment #2 Survival (%)	Experiment #2 No. Mut. per 10^8
Control	—	—	7.7	—	10.2
Exp.	1 hr.	40	5.8	34	10.1
Exp.	2 hr.	11	8.1	7	8.6
Exp.	3 hr.	1.1	7.2	1.1	11.2
Exp.	4 hr.	0.2	6.9	0.1	9.9

99% of the exposed bacteria were killed in about three hours by a 1% solution in distilled water. Table 5 gives the results of two experiments with methyl green, in which the survival was as low as 0.2%, with no increase in the proportion of mutants among the treated bacteria. These results were confirmed by repeated tests. Within the limits of the sensitivity of the technique, which permits the detection of induced mutants under conditions resulting in the destruction of 99.9% of the treated cells, methyl green seems to show no mutagenic activity.

3. *Acriflavine*

Neutral acriflavine, or euflavine, is an acridine dye used therapeutically as a bacteriostatic agent. McIlwain (1941) has shown that the inhibition of bacterial division caused by acriflavine can be reversed by adding polymerized yeast or thymus nucleic acids. He also showed, *in vitro* experiments, that stable complex salts are formed by acriflavine and nucleates. There is again no basis for assuming specificity in these effects, since a number of other compounds, including certain amino acids, were also found to be effective in reversing the inhibition brought about by acriflavine.

TABLE 6. ZERO POINT MUTATIONS INDUCED BY ACRIFLAVIN

Conc. of Acriflavin	Time of Exposure	Survival (%)	No. of Induced Mutations/10^8 Survivors
.02	3 hr.	50.4	8.0
.01	$2\frac{1}{2}$ hr.	12.8	16.5
.01	4 hr.	6.5	54.1
.05	2 hr.	2.5	24.7
.05	2 hr.	1.1	60.6
.05	3 hr.	0.4	51.3
.05	4 hr.	0.08	400
.05	$4\frac{1}{2}$ hr.	0.02	420
.05	4 hr.	0.005	1540
.05	4 hr.	0.0007	4000

Table 6 gives a summary of results obtained with acriflavine. Although reproducible results can be obtained if all factors are carefully standardized, acriflavine presents certain difficulties which stand in the way of quantitative investigation. In the presence of large numbers of bacteria, some of the dye precipitates at high concentrations, and the precipitate goes gradually into solution as the tubes are incubated. Thus it is likely that the effective concentration changes during the exposure. This difficulty can be overcome to some extent by using low concentrations and longer periods of exposure. Results with acriflavine consistently indicate an increase in the proportion of mutants among survivors of treated cultures, and suggest that this compound is active in inducing mutations.

Demerec *et al* (1946) tested the action of acriflavine on Drosophila, prior to the experiments reported here, and obtained negative results. More recently, Demerec (unpub.) has retested acriflavine, using an improved method of administering the chemical, and has found that it is active in inducing lethal mutations in Drosophila.

DISCUSSION

The experiments described above have indicated that exposure to three out of the four chemicals tested, at concentrations sufficiently toxic to kill all but a small fraction of the treated bacteria, results in a heightened proportion of mutants among the survivors. Since B/r/1 was found to be no more resistant than the nonmutant strain B/r to each of the chemicals, it has been concluded that simple selection is not responsible for this effect. Sodium desoxycholate, pyronin and acriflavine are therefore regarded as mutagenic chemicals, although the results for the latter two compounds are only preliminary. Methyl green is apparently unable to induce mutations to phage-resistance.

Although a single specific phenotype, resistance to bacteriophage T1, was the genetic character used in these experiments, it is likely that at least two separate mutations were involved, since B/r/1 mutants are known to fall into two distinct classes, differentiated by such secondary characters as colony size, cross resistance to another bacteriophage and growth factor requirements. No attempt was made to separate the two mutant types in these experiments, and the frequencies observed are probably the sums of the two independent mutations.

Because of the lack of specificity in the chemical action of the compounds tested, no attempt can be made at present to relate their effectiveness as mutagenic agents to the properties for which they were selected. The high incidence of positive results obtained, in 3 out of 4 compounds examined, is also difficult to interpret at the present time. Further experiments are required to determine whether these results are due to a particularly fruitful or fortunate basis of selecting chemicals, or, as appears more likely, whether mutagenic action may prove to be more common among biologically active compounds than has hitherto been believed.

Although bacteria are becoming increasingly useful as material for genetic investigations, it is still necessary to be cautious in generalizing results obtained exclusively in bacterial studies. Thus, the fact that sodium desoxycholate and acriflavine appear to induce mutations in Drosophila as well as in *E. coli* is an important contribution toward the validation of the techniques used in these experiments. In addition to confirmation provided by tests on other organisms, the use of other mutations in *E. coli*, entirely independent of the phage-resistance system, would constitute a valuable check on results obtained with nonspecific mutagens. Experiments to

determine the effects of chemicals on the frequency of reverse mutations from biochemically deficient mutants of *E. coli* are being planned with this need in mind.

The use of phage-resistance in *E. coli* as material for the investigation of chemically induced mutations offers certain unique possibilities, in addition to its value as a screening test. The analysis of dosage-effect relations, as well as the quantitative investigation of the delayed expression of induced mutations characteristic of radiations and mustard gas, can be carried out with this material. It may also be possible to approach another interesting aspect of induced mutations, the comparison of the effectiveness of mutagenic chemicals on resting and dividing cells. It must be remembered, however, that bacteria are relative newcomers to the laboratory of the geneticist, and that a longer and more intimate acquaintance may be required to establish the reliability of these organisms as tools for the study of the broader problems of heredity and mutation.

Summary

Four compounds were tested for mutagenic activity in *E. coli*. The techniques used involved suspending resting bacteria in solutions of the chemicals, under conditions resulting in the death of about 99% of the treated cells. The number of mutants resistant to a bacteriophage, T1, per 10^8 survivors of a treated culture was compared with the number per 10^8 untreated bacteria from the same culture. Only mutations expressed phenotypically before division of the exposed individuals were detected.

The number of mutants per 10^8 survivors was found to be higher in cultures treated with sodium desoxycholate, pyronin and acriflavine than in untreated samples of the same cultures. No such increase was obtained with methyl green. Since mutants and nonmutants were shown to be equally sensitive to the toxic action of each of the compounds, it has been concluded that selection is not responsible for these results. Sodium desoxycholate, pyronin and acriflavine are considered, therefore, to be mutagenic, while methyl green is not.

The number of mutations induced by sodium desoxycholate is directly proportional to the time of exposure to a 5% solution of this compound.

Acknowledgments

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Discussion

Hotchkiss: In examining the most interesting results of Dr. Witkin, I have been struck by the fact that the *actual recovery* of mutants from a fixed number of original cells, which is the count actually observed in the laboratory, is maximal in the high survival range and decreases markedly as the percentage of total survivors drops. Since the number of mutants does not however decrease to the extent that the total population does, it tends to rise rather considerably, if calculated over to the basis of survivors. It has been recognized by the author that to view this latter calculated value as a "mutation rate" involves the assumption that the phage-resistant mutants do not have increased survival value in solutions of the chemical mutagens used. Indeed for this reason, trouble was taken to demonstrate that stock phage-resistant mutants do not survive longer than non-mutants in desoxycholate.

It appears, however, that the actual mutants isolated after desoxycholate, pyronin or acriflavine treatment were not tested for resistance to these chemicals. It should be pointed out that only a modest degree of chemical-resistance in only a portion of the isolated mutant clones would allow the observed increased proportion of mutants among the survivors.

To bring out this point more clearly I may be permitted perhaps to recalculate one of the tables from the lantern slide dealing with acriflavine-treated cultures. The other tables would give very similar results. From the figures given it is possible to recalculate the actual yield of mutants observed from each 10^8 bacteria of the original culture. While this column has to be calculated from another in this instance the point must be stressed that it was originally an observed part of the experimental data and tabulating it involves no particular assumptions.

Per cent survival	Mutants per 10^8 survivors	Corrected for estimated "background"	Yield of mutants per 10^8 original cells	Per cent of Yield expressed as original mutants
100	0	5	5	(100)
50	8	13	6.5	ca.130
6.5	51	56	3.6	72
1	61	66	0.7	14
0.02	420	425	0.085	1.7
0.005	1540	1545	0.077	1.5
0.0007	4000	4000	0.028	0.6

It will be noted that, considered from this point of view, the most striking production of mutants was in the low-killing range where an increase of some 30% was observed. Detected as it presumably was in counts of some one or two hundred colonies, this increase represents at present the only positive indication, free of assumptions, that new mutants have been produced. The particular figure is of course rather dependent upon the value taken for the background content of mutants; what was understood to be an average figure was used here. In any case, where the killing has been greater, the "yield" of mutants has also decreased well below the background value.

Although several theories or explanations come to mind to account for the findings, it should be pointed out that these comments arise as it were from experimental considerations only and are suggested mainly as a more conservative way of expressing the data. When it has been demonstrated to what degree, if any, representative mutants recovered from the chemical mutagen solutions are resistant to that mutagen, then it will be more safe to calculate the actual yield of mutants, dead and alive, produced from 10^8 original cells.

Finally, it may be pointed out that there is no reason to suppose that mutations induced by chemicals need show randomness with repect either to the individual cells affected within a population or to the characters, genetic or somatic, affected within a given cell. Once a particular cell, by virtue of its permeability, physiological state, internal pH, or other inherent or transient property, has accumulated in its interior sufficient chemical mutagen to suffer a mutation at one locus, we would indeed expect that many other changes might become manifest. This picture would furnish one plausible basis for the conservative viewpoint that only a moderate amount of chemically-induced mutation has taken place, and that this has been to some degree associated with temporary or inheritable resistance to the chemicals, a finding not to be expected to any great extent in spontaneous or irradiation-induced mutants.

WITKIN: Dr. Hotchkiss has emphasized the fact that the absolute recovery of mutants falls off, except in the range of high survival, as the exposure to the chemical is extended. This is true not only for the chemicals studied in these experiments, but for radiations as well. An absolute increase of mutants is to be expected only in the case of nontoxic mutagens, or in the case of agents whose mutagenic activity is relatively much greater than its toxicity. No such case is known as yet, and it seems unlikely, at least for mutagens acting in an aspecific manner, that such cases are theoretically possible, since induced lethal mutations may be expected to exceed, as a class, any single phenotype under consideration. Nevertheless, the fact that absolute increases are not observed, except where survival is high, does create the necessity for caution in interpreting the results.

First of all, it can be pointed out that, for each of the three chemicals considered to be mutagenic, absolute increases in the recovery of mutants were observed under special circumstances. In the case of acriflavin and pyronin, the actual number of mutants recovered from a given number of original bacteria increases significantly when the killing does not exceed 50% to 60%. In the case of sodium desoxycholate, it is possible to use low concentrations where the maximal killing is reached by 24 hours, and there is no further killing between 24 and 48 hours of exposure. The number of mutants, however, does increase during this period, resulting in actually higher mutant counts on the experimental

plates at 48 hours, starting with a sample of the same size. Thus, for each chemical it is possible to show that new mutants do actually appear.

Dr. Hotchkiss suggests that the induced mutants may differ from the spontaneous mutants in resistance to the chemical used to induce them. In the case of desoxycholate, where there is a linear relation between the number of induced mutations and the time of exposure to the chemical, in spite of the fact that the rate of killing changes markedly during the period of exposure, it is difficult to see how selection can be involved. If the increase in number of mutants were due in large measure to the selective survival of a few mutants induced early in the treatment, surely the rate of increase in the proportion of mutants with time could not be totally independent of the survival curve.

Dr. Hotchkiss suggests that the mutant colonies actually obtained after treatment with the chemical should be compared with the nonmutant stock with respect to resistance to the chemical. It seems to me that this would not constitute a critical test of the hypothesis Dr. Hotchkiss has raised. First of all, a certain amount of the chemical is carried over onto the plates, and then mutant colonies develop in the presence of a very low concentration of the chemical, negligible in terms of toxic or mutagenic activity, but possbily sufficient to permit selection during the growth of the colony of spontaneous variants better able to withstand the action of the substance. Thus, to find that the mutant colonies obtained after exposure to the chemical are more resistant to its toxic action than the untreated nonmutant stock, is not a fair test of the situation before the development of the colony on the plate. Should the mutant colonies prove no more resistant to the action of the chemical than the untreated non-mutant stock, the possibility of a temporary resistance, as Dr. Hotchkiss has suggested, can be raised. I cannot at present see any way of testing this possibility critically, since it is based on the state of a recently induced mutant, during treatment with the chemical, and a transient resistance to the chemical can be postulated. One possible way to avoid considerations of this kind might be to start out by developing a strain which is maximally resistant to the chemical to begin with, and use this strain throughout.

The best evidence that new mutations are in fact appearing throughout the exposure, to my mind, will be based upon comparison of survival curves and curves of induced mutations as a function of exposure time. If, as has been found for desoxycholate, the proportion of mutants increases at a constant rate, regardless of changes in killing rate, the possibility that recently induced mutants survive selectively will become quite remote.

Kurnick: Dr. Witkin has expressed some surprise at the fact that 3 out of 4 tested chemicals proved mutagenic in her experiments and suggested that it will be found that mutagenicity is a common property of chemical reagents. This appears to be a not unlikely prognostication. While not wishing to deny the possibility of direct chemical action to induce mutations, I should like to present an alternative hypothesis here.

Dr. Witkin searches only for a specific mutation-resistance to T1 phage—not random mutations. She observes that this mutation is present spontaneously in her controls in 5 to 10 per 10^8 bacilli. It is not improbable that this mutant contains a specific mutagenic substance similar to that isolated by Avery for pneumococcic strains and by Boivin for a colon bacillus strain. Dr. Witkin has observed that the mutation effect of a given chemical corresponds more nearly to its killing effect than to any other factor, such as concentration of the chemical or the time of exposure. It is obvious that if, as she has shown, the mutant and the original strains are equally sensitive to the lethal effect of the drugs used, the higher the percentage of lethality, the greater will be the chance of destruction of some of the 5 to 10 spontaneous mutants per 10^8 bacteria. The autolysis of the killed mutant (or penetration through its wall whose permeability is apt to differ from the living cell) will free the specific mutator substance (for T1 phage resistance) into the medium where it may cause the specific, directed mutation of other organisms. The nearer the killing rate approaches 100%, the higher will be the concentration of this mutator substance, and so the higher the mutation rate.

Dr. Witkin has observed that after about 99.9% of the bacteria have been killed, the killing rate approaches zero, but the production of mutations continues. She suggests that this may mean that the lethal and mutagenic properties of her chemicals are unrelated, and that the 0.1% of surviving cells, while no longer susceptible to the lethal effect are still susceptible to the mutagenic effect of the drug. If the hypothesis presented herein were correct, we could readily account for the continued occurrence of mutations in the surviving bacteria by the fact that they are continuously exposed to the liberated mutator of the killed spontaneous mutants and so continue to undergo directed mutation.

Of the 4 chemicals studied by Dr. Witkin, only methyl green was inert as a "mutagen," although its lethality in the concentrations used was comparable to that of the others. Now if, in fact, as is indicated by Boivin and Avery, desoxyribonucleic acid is an essential component (perhaps the only component, but this argument is not germaine to this discussion), a chemical which combined specifically with this nucleic acid might inactivate the mutator substance. Methyl green is such a compound (as opposed to pyronin, for example). Thus, we may account for the lethality of this compound without the expected production of mutations.

On the other hand, some chemicals, which might

themselves not inactivate the mutator substance, might nevertheless render it ineffective by altering the cell surface of the bacteria so as to deny access of the mutator substance to the interior of the cell. Such a reagent may be chloroform, which Dr. Beale has found ineffective as a mutagen despite its lethal effect.

Thus, the induction of mutations by chemicals which do not themselves directly interact with the gene would be a function of their lethality, the degree of interaction between the chemical reagent and the liberated mutator substance, and the degree to which the permeability of the cell wall is altered. In this manner we may account for differences in "mutagenicity" of compounds with identical killing rates.

This hypothesis is susceptible of experimental test in several ways. I shall suggest two such experiments. One would be to test (a) the effect of lethal reagents which do not induce mutations (particularly methyl green) on the mutator substances of Boivin and Avery before addition to the culture substrates to determine whether inactivation does in fact occur, and (b) the effect of adding the chemical (particularly chloroform) to the appropriate culture before adding Boivin's or Avery's substance to determine whether a reagent which does not itself inactivate the mutator substance but nevertheless fails to produce mutations, prevents the effect of the mutator substance by its action in the otherwise susceptible cells. Another would be to centrifuge off the surviving bacteria after 99.9% had been killed, wash them several times in water and then resuspend them in a medium containing the chemical under investigation (let us say pyronin). Controls would consist of bacteria left in the original medium and bacteria resuspended in water. If the bacteria resuspended in a fresh solution of the reagent and those resuspended in water show a significantly lower rate of continued mutation than those permitted to remain in the original suspension (provided the killing rate remained near zero in all samples), one could conclude that the mutagenic factor was not the chemical reagent, but rather some material which was present in the first supernate alone—this could only be derived from the cultures themselves. If, on the other hand, mutations continued to occur in all three test samples at the same rate, the conclusion would again be that the mutagenicity was probably not a direct function of the chemical (since one suspension is resuspended in water), but that the mutator substance was adsorbed to the washed bacteria or had a significant lag time between its action on the bacteria and the emergence of the mutant. The third result which might occur, namely that the sample resuspended in a fresh solution of the chemical showed a continued mutation rate equal to that of the original suspension and greater than that of the suspension in water, would disprove the hypothesis I have proposed above and demonstrate the capacity of the chemicals themselves to produce mutations, presumably by direct attack on the gene.

I do not by any means, I repeat, wish to imply that chemical mutations due to the direct action of a chemical reagent on the gene do not occur. Quite the contrary, there is good reason to believe that such effects are possible. I wish only to interject a word of caution in interpreting the induction of mutations by a considerable number of reagents, particularly when working with such material as bacteria where the opportunity for interaction between large numbers of organisms exists. This pitfall may be avoided by suitable controls, as suggested above, to distinguish between direct interaction with the gene and the indirect effect due to autolysis.

WITKIN: It is, of course, possible that phage-resistant mutants of strain B/r contain specific mutator substances, similar to the transforming factors of Avery and Boivin. The addition of filtrates of heat-killed cultures of B/r/1, however, does not increase the number of resistant mutants in cultures of B/r. Numerous other attempts to detect evidence for transformation in this system have failed. This, in addition to the fact that the number of induced mutants obtained is independent of the background number, over a range of one to fifty per 10^8 bacteria, seems to me to render Dr. Kurnick's hypothesis unlikely.

BRYSON: In the event that we continue to find numerous and unrelated chemicals that are able to induce mutation, it may be of particular interpretive value to study with care those substances that are *not* mutagenic. It would also be desirable that more simple chemical substances such as inorganic salts be surveyed as mutagens, even as Drs. Greenstein, Carter and Chalkley have surveyed them for effect on enzymatic degradation of nucleic acid. One might then bring to bear what is known of the effects of ions on cells, and compare mutagenesis with what has been established about permeability and relative toxicity of various materials whose fate as reactants in living systems has already been the subject of extensive biochemical investigations. The problem of toxicity itself presents a difficulty in the classification of chemical mutagens since toxicity may act as a limiting factor in exploring mutagenic potential. For example, we have found that the induction of mutations of phage resistance in *E. coli* by bis-beta-chloroethylamine hydrochloride is most readily performed on a strain of cells that has been through twelve consecutive exposures to the inducing agent with repeated selection of survivors for resistance to the chemical. The number of zero point mutations induced by nitrogen mustard in stock B_{12}/M at a survival level of 0.007% is 240 per 10^8. It is always possible that a chemical like methyl green that Dr. Witkin has described as negatively mutagenic could be made to induce mutations in a strain of cells

selected for resistance to its toxic effects and therefore capable of surviving in relatively high concentrations.

No attempt has been made to approach a comparable killing value with another mutagenic agent (Zephiran chloride) because this quaternary ammonium compound shows a selective toxic effect against B/1 at high concentrations, a process which incidentally would tend to make the detection of mutations a more difficult process. An experiment using 1:14,000 aq. Zephiran chloride (a non-selective concentration) is shown in Table 1. A definite mutagenic effect is observed if periodic sampling of the same culture is performed.

TABLE 1. PERIODIC ASSAY OF CELLS INCUBATED WITH ZEPHIRAN CHLORIDE AT 37° C. AND TESTED FOR 0 POINT MUTATIONS TO PHAGE RESISTANCE

Time of Assay	Bacteria per cc.	B/1 per 10^8 bacteria	Per cent survival	Zero point mutants
0	5.6×10^{10}	$4.2/10^8$	—	—
3 min.	2.2×10^{10}	$8.1/10^8$	39%	$3.9/10^8$*
10 min.	2.0×10^{10}	$8.6/10^8$	36%	$4.4/10^8$
30 min.	1.1×10^{10}	$13.3/10^8$	20%	$9.1/10^8$
100 min.	6.4×10^9	$21.1/10^8$	11%	$16.9/10^8$

* $8.1/10^8-4.2/10^8$.

In another experiment yielding 19% survival the zero point mutations numbered 24 per 10^8.

The intergeneric variation in sensitivity of microorganisms to toxic agents is perhaps of more general importance than experimentally induced interstrain differences as a factor to be kept in mind when screening of possible mutagens is contemplated. Unless one adopts the relatively extreme position that cell death in the presence of chemicals is the consequence of induced lethals it is not unduly speculative to assume that cells highly resistant to deleterious chemical effects on extragenic processes will be best suited for a demonstration of mutagenesis. If analysis of toxicity is to be performed as a differential survival test it may also be necessary with some chemicals to extend the tests over the killing range included within limits of the experiment.

As Dr. Witkin has observed, the span of our experience with induced phage resistance as a genetic tool has not been of sufficient length to evaluate its place in the general scheme of experimentally induced mutations in more complex biological systems. Investigators using the method may find themselves in the paradoxical situation that each new success contributes to a failure, that is, to the widening of a gap between mutagenesis of microorganisms and of higher forms of life. This would inevitably decrease the value of bacterial studies in interpreting mutation among higher organisms. Until other mutations in bacteria have been studied by similar methods and until the growing list of chemical mutagens has been used on more familiar genetic material and placed within some kind of quantitative and qualitative limits, it will be impossible to judge the real significance of Dr. Witkin's most stimulating and capable study.

HERSKOWITZ: The work of Dr. Witkin demonstrates a facile bacterial method for identifying certain groups of chemicals as mutagens. She has already pointed out that by using this technique negative mutagenic action by a chemical is not conclusively demonstrated. However, it may well be that those chemicals screened out by the bacterial technique as being non-mutagenic will be important in the long run. Therefore, it seems advisable to supplement this technique with others which may permit a more sensitive test for mutagenic action.

For the detection of the action of a chemical on nucleic acids and nucleoproteins the use of Drosophila sperm seems most suitable. There are several reasons for this. In spermatozoa there are a minimum of cytoplasmic substances which might interfere with a chemical affecting genic nucleoprotein. Moreover, even though a chemical is not specific for nucleoprotein it may yield positive results more readily in sperm than in other cells. There is excellent evidence from genetic and irradiation experiments that genic nucleoprotein may be drastically changed without killing the sperm cell; this permits the utilization of such sperm for fertilization with the subsequent detection of lethal genes and chromosome rearrangements in addition to other types of inherited changes. In Drosophila it is also possible accurately to localize inherited changes in the chromosomes. Dr. Demerec no doubt had these advantages in mind when he developed the aerosol technique.

As Dr. Witkin has mentioned, there is no conclusive proof that aerosols always reach the spermatozoa in the testes. Again, negative mutagenic results with a chemical may be misleading. Therefore, a technique which would directly treat Drosophila sperm with chemicals is highly desirable. Accordingly, an investigation was initiated to discover if chemicals could be injected into the vagina of adult females, which would then copulate, and thereby expose the sperm to the respective chemicals.

A vaginal douche technic was successfully worked out, and is described in detail in a recent published note (Herskowitz, Evolution *1:* 111-112, 1947). The method was tried with methyl bis amine hydrochloride at concentrations of 0.2% and 10.0%, with positive results.

The data establish the practicality of the vaginal douche technique for the detection of chemical mutagens. There are two advantages of this method. Low concentrations of mutagens may be effective

because of the direct contact of the chemical with the sperm; high concentrations of chemicals can be used without killing the organism, since a localized part of the body rather than the whole individual is treated. These advantages point to the possibility of a chemical analysis of the processes leading to gene changes as well as the analysis of the processes involved in direct changes in genic nucleoprotein. With such objectives in mind, the search for and study of the mutagenic activity for many compounds would most efficiently be investigated by using the bacterial or fungus method first, then the use of the aerosol method, and finally, if necessary, the vaginal douche technic.

ZAMENHOF: I wonder if Dr. Witkin would be willing to comment on *qualitative* differences between spontaneous mutations and mutations induced by chemicals, in addition to the *quantitative* ones. As Dr. Witkin has pointed out, the spontaneous mutations do not seem to occur in the non-dividing cells (see also Zamenhof, Genet. Soc. Rec. *13:* 41-42, 1944). The mutation ratio in this case can therefore be defined as the number of mutations per cell division. On the other hand, the mutations induced by chemicals seem to take place even in the absence of cell divisions; in this case the mutation ratio can be defined as the number of mutations per cell per unit of time. Thus the two phenomena seem to be different. What is your opinion on this subject?

WITKIN: The fact that mutations can be induced in resting bacteria by radiations and chemicals, whereas spontaneous mutations seem to occur only in dividing cells, certainly suggests an important difference between induced and spontaneous mutations, although the end product of the two processes, the mutated genes, need not necessarily differ in a fundamental way. Chemical or mechanical errors of duplication, or slips in some metabolic cycle inactive in resting cells, may be the only natural circumstances capable of bringing about mutations. Thus, the failure of spontaneous mutation to occur in resting cells may result from the relatively inert and quiescent state of the genic material, and of the metabolic activities associated with cell division, rather than to a greater degree of inherent stability. Zero point mutations may be due to the direct action of chemicals or radiations on the gene, or more indirectly, to the stimulation of cellular activities normally involved in the production of spontaneous mutations. A careful comparison of the properties of mutants arising spontaneously and by induction may throw some light on this important question.

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